FORM PTO-1390 (Modified) (REV 11-2000) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER	U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR
57127 (46342)	10/070240

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/JP00/05685	24/08/00	27/08/99

TITLE OF INVENTION
NOVEL G PROTEIN-COUPLED RECEPTOR PROTEIN AND DNA THEREOF

APPLICANT(S) FOR DO/EO/US
TAKUYA WATANABE, ET AL.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include itens (5), (6), (9) and (24) indicated below.
4. ☐ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
   a. ☒ is attached hereto (required only if not communicated by the International Bureau).
   b. ☐ has been communicated by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☒ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. ☒ is attached hereto.
   b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
   a. ☒ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☐ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
10. ☐ An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).
11. ☐ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☒ A copy of the International Search Report (PCT/ISA/210).

Items 13 to 20 below concern document(s) or information included:

13. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☒ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☒ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
20. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
21. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
22. ☒ Certificate of Mailing by Express Mail
23. ☒ Other items or information:

Associate Power of Attorney; PCT/RO/101; PCT/RO/106; PCT/IB/301; PCT/BA/220: PCT/IPEA/401; PCT/IPEA/402; PCT/IPEA/408; PCT/IPEA/416; PCT/IB/332; PCT/IB/308; PCT/RO/105; PCT/IB/304

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
10/070240	PCT/JP00/05685	57127 (46342)

24. The following fees are submitted:.		CALCULATIONS PTO USE ONLY	
BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :			
☐ Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO	$1040.00		
☐ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO	$890.00		
☒ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO	$740.00		
☐ International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4)	$710.00		
☐ International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4)	$100.00		
ENTER APPROPRIATE BASIC FEE AMOUNT =		$740.00	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).		$0.00	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	14 - 20 =	0	x $18.00	$0.00	
Independent claims	1 - 3 =	0	x $84.00	$0.00	
Multiple Dependent Claims **(check if applicable)**.			☒	$280.00	
TOTAL OF ABOVE CALCULATIONS =				$1,020.00	
☐ Applicant claims small entity status. See 37 CFR 1.27). The fees indicated above are reduced by 1/2.				$0.00	
SUBTOTAL =				$1,020.00	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)). +				$0.00	
TOTAL NATIONAL FEE =				$1,020.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) **(check if applicable).** ☒				$40.00	
TOTAL FEES ENCLOSED =				$1,060.00	
				Amount to be: refunded	$
				charged	$

a. ☒ A check in the amount of **$1,060.00** to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. _____ in the amount of _____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. **04-1105** A duplicate copy of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

David G. Conlin, Esquire
Dike, Bronstein, Roberts & Cushman
Intellectual Property Practice Group of
Edwards & Angell, LLP
PO BOX 9169
Boston, MA 02209

Dianne Rees
SIGNATURE

Dianne Rees, Ph.D.
NAME

45,281
REGISTRATION NUMBER

27 February 2002
DATE

JC18 Rec'd PCT/PTO 27 FEB 2002

TRANSMITTAL LETTER TO THE UNITED STATES RECEIVING OFFICE

PTO-1382 (Rev. 4-1995) (Modified) - PCTUS2.FRP /REV03

Date	27 February 2002
International Application No.	PCT/JP00/05685
Attorney Docket No.	57127 (46342)

I. Certification under 37 CFR 1.10 (if applicable)

EL933048924US	10/070240
Express Mail mailing number	27 February 2002 Date of Deposit

I hereby certify that the application/correspondence attached hereto is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to Assistant Commissioner for Patents, Washington, D.C. 20231.

Signature of person mailing correspondence	Fatima H. DeArruda Typed or printed name of person mailing correspondence

II. ☐ New International Application

TITLE	NOVEL G PROTEIN-COUPLED RECEPTOR PROTEIN AND DNA THEREOF	Earliest priority date (Day/Month/Year): 27/08/99

SCREENING DISCLOSURE INFORMATION: In order to assist in screening the accompanying international application for purposes of determining whether a license for foreign transmittal should and could be granted and for other purposes, the following information is supplied. (Note: check as many boxes as apply):

A. ☒ The invention disclosed was **not** made in the United States.

B. ☒ There is no prior U.S. application relating to this invention.

C. ☐ The following prior U.S. application(s) contain subject matter which is related to the invention disclosed in the attached international application. *(NOTE: priority to these applications may or may not be claimed on form PCT/RO/101 (Request) and this listing does not constitute a claim for priority).*

application no.		filed on	
application no.		filed on	

D. ☐ The present international application ☐ is identical ☐ contains less subject matter than that found in the prior U.S. application(s) identified in paragraph C.

E. ☒ The present international application ☐ contains additional subject matter not found in the prior U.S. application(s) identified in paragraph C. above. The additional subject matter is found on pages ____ and ☐ **DOES NOT ALTER** ☐ **MIGHT BE CONSIDERED TO ALTER** the general nature of the invention in a manner which would require the U.S. application to have been made available for inspection by the appropriate defense agencies under 35 U.S.C. 181 and 37 CFR 5.1. See 37 CFR 5.15

III. ☐ A Response to an Invitation from the RO/US. The following document(s) is (are) enclosed:

A. ☐ A Request for An Extension of Time to File a Response

B. ☐ A Power of Attorney (General or Regular)

C. ☐ Replacement pages:

pages		of the request (PCT/RO/101)	pages		of the figures
pages		of the description	pages		of the abstract
pages		of the claims			

D. ☐ Submission of Priority Documents

Priority document		Priority document	

E. ☐ Fees as specified on attached Fee Calculation sheet form PCT/RO/101 annex

IV. ☐ A Request for Rectification under PCT 91 ☐ **A Petition** ☒ **A Sequence Listing Diskette**

V. ☐ Other (please specify):

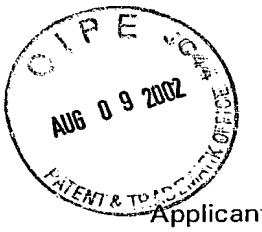
The person signing this form is the:

☐ Applicant	Dianne Rees, Ph.D.
☒ Attorney/Agent (Reg. No.) 45,281	Typed name of signer
☐ Common Representative	Dianne Rees Signature

EXPRESS MAIL LABEL NO:
EL933049045 US

#4

Atty. Dkt. No. (46342) 57127



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Takuya WATANABE, *et al.*

Title: NOVEL G PROTEIN-COUPLED RECEPTOR PROTEIN AND DNA THEREOF

Appl. No. 10/070,240

Filing Date: February 27, 2002

Examiner: Not Yet Assigned

Art Unit: Not Yet Assigned

AMENDMENT IN RESPONSE TO NOTICE UNDER 37 CFR §§1.821-825

BOX SEQUENCE
Commissioner for Patents
Washington, D.C. 20231

Sir:

In response to the Notice to Comply With Requirements for Applications Containing Sequence Disclosures mailed June 12, 2002, please amend the application as follows:

In the Specification:

Please delete the paragraph on page 10, lines 6-9, and replace it with the following paragraph:

Fig. 1 shows the base sequence of DNA (SEQ ID NO: 2) encoding the human brain-derived protein (ZAQC) (SEQ ID NO: 1) of the present invention obtained in Example 1, and the amino acid sequence deduced from the base sequence (following to Figure 2).

Please delete the paragraph on page 10, lines 10-14, and replace it with the following paragraph:

Fig. 2 shows the base sequence of DNA (SEQ ID NO: 2) encoding the human brain-derived protein (ZAQC) (SEQ ID NO: 1) of the present invention obtained in Example 1, and the amino acid sequence deduced from the base sequence (continued from Figure 1 and following to Figure 3).

Please delete the paragraph on page 10, lines 15-18, and replace it with the following paragraph:

Fig. 3 shows the base sequence of DNA (SEQ ID NO: 2) encoding the human brain-derived protein (ZAQC) (SEQ ID NO: 1) of the present invention obtained in Example 1, and the amino acid sequence deduced from the base sequence (continued from Figure 2).

Please delete the paragraph on page 10, lines 19-22, and replace it with the following paragraph:

Fig. 4 shows the base sequence of DNA (SEQ ID NO: 3) encoding the human brain-derived protein (ZAQT) (SEQ ID NO: 1) of the present invention obtained in Example 1, and the amino acid sequence deduced from the base sequence (following to Figure 5).

Please delete the paragraph on page 10, lines 23-27, and replace it with the following paragraph:

Fig. 5 shows the base sequence of DNA (SEQ ID NO: 3) encoding the human brain-derived protein (ZAQT) (SEQ ID NO: 1) of the present invention obtained in Example 1, and the amino acid sequence deduced

from the base sequence (continued from Figure 4 and following to Figure 6).

Please delete the paragraph on page 10, lines 28-31, and replace it with the following paragraph:

Fig. 6 shows the base sequence of DNA (SEQ ID NO: 3) encoding the human brain-derived protein (ZAQT) (SEQ ID NO: 1) of the present invention obtained in Example 1, and the amino acid sequence deduced from the base sequence (continued from Figure 5).

Please delete the paragraph on page 11, lines 1-3, and replace it with the following paragraph:

Fig. 9 shows the amino acid sequence of MIT1 (SEQ ID NO: 32), Human type ZAQ ligand precursor peptide (A type) (SEQ ID NO: 33) and Human type ZAQ ligand precursor peptide (G type) (SEQ ID NO: 34).

REMARKS

Applicants believe that the present application is now in condition for allowance. Favorable reconsideration of the application as amended is respectfully requested.

The Examiner is invited to contact the undersigned by telephone if it is felt that a telephone interview would advance the prosecution of the present application.

Atty. Dkt. No. (46342) 57127

Respectfully submitted,

Date August 9, 2002

By Dianne Rees

EDWARDS & ANGELL, LLP
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101 Federal Street
Boston, MA 02110-1800
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Dianne M. Rees
Attorney for Applicant
Registration No. 45,281

Should additional fees be necessary in connection with the filing of this paper, or if a petition for extension of time is required for timely acceptance of same, the Commissioner is hereby authorized to charge Deposit Account No. 04-1105 for any such fees; and applicant(s) hereby petition for any needed extension of time.

Atty. Dkt. No. (46342) 57127

MARKED UP VERSION ATTACHED TO AMENDMENT IN

SERIAL NO. 10/070,240

Marked up version of the paragraph starting at page 10, lines 6-9 is below:

Fig. 1 shows the base sequence of DNA (SEQ ID NO: 2) encoding the human brain-derived protein (ZAQC) (SEQ ID NO: 1) of the present invention obtained in Example 1, and the amino acid sequence deduced from the base sequence (following to Figure 2).

Marked up version of the paragraph starting at page 10, lines 10-14 is below:

Fig. 2 shows the base sequence of DNA (SEQ ID NO: 2) encoding the human brain-derived protein (ZAQC) (SEQ ID NO: 1) of the present invention obtained in Example 1, and the amino acid sequence deduced from the base sequence (continued from Figure 1 and following to Figure 3).

Marked up version of the paragraph starting at page 10, lines 15-18 is below:

Fig. 3 shows the base sequence of DNA (SEQ ID NO: 2) encoding the human brain-derived protein (ZAQC) (SEQ ID NO: 1) of the present invention obtained in Example 1, and the amino acid sequence deduced from the base sequence (continued from Figure 2).

Marked up version of the paragraph starting at page 10, lines 19-22 is below:

Fig. 4 shows the base sequence of DNA (SEQ ID NO: 3) encoding the human brain-derived protein (ZAQT) (SEQ ID NO: 1) of the present invention obtained in Example 1, and the amino acid sequence deduced from the base sequence (following to Figure 5).

Marked up version of the paragraph starting at page 10, lines 23-27 is below:

Fig. 5 shows the base sequence of DNA (SEQ ID NO: 3) encoding the human brain-derived protein (ZAQT) (SEQ ID NO: 1) of the present invention obtained in Example 1, and the amino acid sequence deduced from the base sequence (continued from Figure 4 and following to Figure 6).

Marked up version of the paragraph starting at page 10, lines 28-31 is below:

Fig. 6 shows the base sequence of DNA (SEQ ID NO: 3) encoding the human brain-derived protein (ZAQT) (SEQ ID NO: 1) of the present invention obtained in Example 1, and the amino acid sequence deduced from the base sequence (continued from Figure 5).

Marked up version of the paragraph starting at page 11, lines 1-3 is below:

Fig. 9 shows the amino acid sequence of MIT1 (SEQ ID NO: 32), Human type ZAQ ligand precursor peptide (A type) (SEQ ID NO: 33) and Human type ZAQ ligand precursor peptide (G type) (SEQ ID NO: 34).

#4.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Atty. Docket No: (46342) 57127

In re patent application of

WATANABE, TAKUYA *et al.*

Serial No. 10/070,240

Filed: February 27, 2002

For: NOVEL G PROTEIN-COUPLED RECEPTOR PROTEIN AND DNA THEREOF

STATEMENT TO SUPPORT FILING AND SUBMISSION IN ACCORDANCE WITH 37 C.F.R. §§ 1.821-1.825

Assistant Commissioner for Patents
Washington, D.C. 20231
Box SEQUENCE

Sir:

In connection with a Sequence Listing submitted concurrently herewith, the undersigned hereby states that:

1. the submission, filed herewith in accordance with 37 C.F.R. § 1.821(g), does not include new matter;

2. the content of the attached paper copy and the attached computer readable copy of the Sequence Listing, submitted in accordance with 37 C.F.R. § 1.821(c) and (e), respectively, are the same; and

3. all statements made herein of their own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United

Serial No. 10/070,240

States Code and that such willful false statements may jeopardize the validity of the application or any patent resulting therefrom.

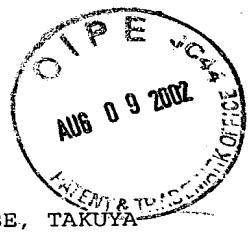
Respectfully submitted,

July 22, 2002
Date

James A. Coburn

HARBOR CONSULTING
Intellectual Property Services
1500A Lafayette Road
Suite 262
Portsmouth, N.H.
800-318-3021

#4



SEQUENCE LISTING

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TERAO, YASUKO
SHINTANI, YASUSHI

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Phe Pro Phe Asn Phe Ser Tyr Ser Asp Tyr Asp Met Pro Leu Asp Glu
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Asp Glu Asp Val Thr Asn Ser Arg Thr Phe Phe Ala Ala Lys Ile Val
    50                  55                  60

Ile Gly Met Ala Leu Val Gly Ile Met Leu Val Cys Gly Ile Gly Asn
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Phe Ile Phe Ile Ala Ala Leu Val Arg Tyr Lys Lys Leu Arg Asn Leu
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Thr Asn Leu Leu Ile Ala Asn Leu Ala Ile Ser Asp Phe Leu Val Ala
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Ile Val Cys Cys Pro Phe Glu Met Asp Tyr Tyr Val Val Arg Gln Leu
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Val Lys Ser Gln Glu Lys Ile Phe Cys Gly Gln Ile Trp Pro Val Asp
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225                 230                 235                 240

Phe Val Gly Pro Val Val Thr Met Thr Leu Cys Tyr Ala Arg Ile Ser
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            260                 265                 270

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Ile Leu Thr Ala Tyr Val Leu Cys Trp Ala Pro Phe Tyr Gly Phe Thr
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305                 310                 315                 320

Leu Thr Ala Phe Tyr Ile Val Glu Cys Ile Ala Met Ser Asn Ser Met
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Phe Lys Lys Ile Met Leu Leu His Trp Lys Ala Ser Tyr Asn Gly Gly
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gat gag gat gtg acc aat tcc agg acg ttc ttt gct gcc aag att gtc   192
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65                  70                  75                  80

ttc atc ttt atc gct gcc ctg gtc cgc tac aag aaa ctg cgc aac ctc   288
Phe Ile Phe Ile Ala Ala Leu Val Arg Tyr Lys Lys Leu Arg Asn Leu
                85                  90                  95

acc aac ctg ctc atc gcc aac ctg gcc atc tct gac ttc ctg gtg gcc   336
Thr Asn Leu Leu Ile Ala Asn Leu Ala Ile Ser Asp Phe Leu Val Ala
            100                 105                 110

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Ile Val Cys Cys Pro Phe Glu Met Asp Tyr Tyr Val Val Arg Gln Leu
        115                 120                 125

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Gln Gln Leu Tyr Tyr Lys Ser Tyr Phe Leu Phe Ile Phe Gly Ile Glu
225                 230                 235                 240

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305                 310                 315                 320

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Phe Lys Lys Ile Met Leu Leu His Trp Lys Ala Ser Tyr Asn Gly Gly
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aag tcc agt gca gac ctg gac ctc aag aca att ggg atg cct gcc acc   1152
Lys Ser Ser Ala Asp Leu Asp Leu Lys Thr Ile Gly Met Pro Ala Thr
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gaa gag gtg gac tgc atc aga cta aaa                              1179
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385                 390
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acc agc ttc ctt tct gtg ctc aac cct cat gga gcc cat gcc act tcc   96
Thr Ser Phe Leu Ser Val Leu Asn Pro His Gly Ala His Ala Thr Ser
             20                  25                  30

ttc cca ttc aac ttc agc tac agc gac tat gat atg cct ttg gat gaa   144
Phe Pro Phe Asn Phe Ser Tyr Ser Asp Tyr Asp Met Pro Leu Asp Glu
         35                  40                  45

gat gag gat gtg acc aat tcc agg acg ttc ttt gct gcc aag att gtc   192
Asp Glu Asp Val Thr Asn Ser Arg Thr Phe Phe Ala Ala Lys Ile Val
     50                  55                  60

att ggg atg gcc ctg gtg ggc atc atg ctg gtc tgc ggc att gga aac   240
Ile Gly Met Ala Leu Val Gly Ile Met Leu Val Cys Gly Ile Gly Asn
 65                  70                  75                  80

ttc atc ttt atc gct gcc ctg gtc cgc tac aag aaa ctg cgc aac ctc   288
Phe Ile Phe Ile Ala Ala Leu Val Arg Tyr Lys Lys Leu Arg Asn Leu
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acc aac ctg ctc atc gcc aac ctg gcc atc tct gac ttc ctg gtg gcc   336
Thr Asn Leu Leu Ile Ala Asn Leu Ala Ile Ser Asp Phe Leu Val Ala
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Ile Val Cys Cys Pro Phe Glu Met Asp Tyr Tyr Val Val Arg Gln Leu
        115                 120                 125

tcc tgg gag cac ggc cac gtc ctg tgc acc tct gtc aac tac ctg cgc   432
Ser Trp Glu His Gly His Val Leu Cys Thr Ser Val Asn Tyr Leu Arg
    130                 135                 140

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Thr Val Ser Leu Tyr Val Ser Thr Asn Ala Leu Leu Ala Ile Ala Ile
145                 150                 155                 160

gac agg tat ctg gct att gtc cat ccg ctg aga cca cgg atg aag tgc   528
Asp Arg Tyr Leu Ala Ile Val His Pro Leu Arg Pro Arg Met Lys Cys
                165                 170                 175

caa aca gcc act ggc ctg att gcc ttg gtg tgg acg gtg tcc atc ctg   576
Gln Thr Ala Thr Gly Leu Ile Ala Leu Val Trp Thr Val Ser Ile Leu
            180                 185                 190

atc gcc atc cct tcc gcc tac ttc acc acc gag acg gtc ctc gtc att   624
Ile Ala Ile Pro Ser Ala Tyr Phe Thr Thr Glu Thr Val Leu Val Ile
        195                 200                 205

gtc aag agc cag gaa aag atc ttc tgc ggc cag atc tgg cct gtg gac   672
Val Lys Ser Gln Glu Lys Ile Phe Cys Gly Gln Ile Trp Pro Val Asp
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Phe Val Gly Pro Val Val Thr Met Thr Leu Cys Tyr Ala Arg Ile Ser
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cgg gag ctc tgg ttc aag gcg gtc cct gga ttc cag aca gag cag atc   816
Arg Glu Leu Trp Phe Lys Ala Val Pro Gly Phe Gln Thr Glu Gln Ile
            260                 265                 270

cgc aag agg ctg cgc tgc cgc agg aag acg gtc ctg gtg ctc atg tgc   864
Arg Lys Arg Leu Arg Cys Arg Arg Lys Thr Val Leu Val Leu Met Cys
        275                 280                 285

atc ctc acc gcc tac gtg cta tgc tgg gcg ccc ttc tac ggc ttc acc   912
Ile Leu Thr Ala Tyr Val Leu Cys Trp Ala Pro Phe Tyr Gly Phe Thr
    290                 295                 300

atc gtg cgc gac ttc ttc ccc acc gtg ttt gtg aag gag aag cac tac   960
Ile Val Arg Asp Phe Phe Pro Thr Val Phe Val Lys Glu Lys His Tyr
305                 310                 315                 320

ctc act gcc ttc tac atc gtc gag tgc atc gcc atg agc aac agc atg   1008
Leu Thr Ala Phe Tyr Ile Val Glu Cys Ile Ala Met Ser Asn Ser Met
                325                 330                 335

atc aac act ctg tgc ttc gtg acc gtc aag aac gac acc gtc aag tac   1056
Ile Asn Thr Leu Cys Phe Val Thr Val Lys Asn Asp Thr Val Lys Tyr
            340                 345                 350

ttc aaa aag atc atg ttg ctc cac tgg aag gct tct tac aat ggc ggt   1104
Phe Lys Lys Ile Met Leu Leu His Trp Lys Ala Ser Tyr Asn Gly Gly
        355                 360                 365

aag tcc agt gca gac ctg gac ctc aag aca att ggg atg cct gcc acc   1152
Lys Ser Ser Ala Asp Leu Asp Leu Lys Thr Ile Gly Met Pro Ala Thr
    370                 375                 380

gaa gag gtg gac tgc atc aga cta aaa                              1179
Glu Glu Val Asp Cys Ile Arg Leu Lys
385                 390
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<211> 36
<212> DNA
<213> Artificial Sequence

<220>
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<220>
<223> Description of Artificial Sequence: Primer

<400> 7
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<210> 8
<211> 29
<212> DNA
<213> Artificial Sequence

<220>
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<210> 9
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<210> 10
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<213> Artificial Sequence

<220>
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<223> Any amino acid

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<222> (13)
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<211> 28
<212> DNA
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<220>
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20 25 30

Pro Leu Gly Arg Glu Gly Glu Glu Cys His Pro Gly Ser His Lys Ile
35 40 45

Pro Phe Phe Arg Lys Arg Lys His His Thr Cys Pro Cys Leu Pro Asn
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Leu Leu Cys Ser Arg Phe Pro Asp Gly Arg Tyr Arg Cys Ser Met Asp
65 70 75 80

Leu Lys Asn Ile Asn Phe
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<210> 21
<211> 86
<212> PRT
<213> Homo sapiens

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Pro Leu Gly Arg Glu Gly Glu Glu Cys His Pro Gly Ser His Lys Val
        35                  40                  45

Pro Phe Phe Arg Lys Arg Lys His His Thr Cys Pro Cys Leu Pro Asn
    50                  55                  60

Leu Leu Cys Ser Arg Phe Pro Asp Gly Arg Tyr Arg Cys Ser Met Asp
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Leu Lys Asn Ile Asn Phe
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<212> PRT
<213> Homo sapiens

<400> 22

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            20                  25                  30

Gly Ala Gly Thr Cys Cys Ala Ile Ser Leu Trp Leu Arg Gly Leu Arg
        35                  40                  45

Met Cys Thr Pro Leu Gly Arg Glu Gly Glu Glu Cys His Pro Gly Ser
    50                  55                  60

His Lys Ile Pro Phe Phe Arg Lys Arg Lys His His Thr Cys Pro Cys
65                  70                  75                  80

Leu Pro Asn Leu Leu Cys Ser Arg Phe Pro Asp Gly Arg Tyr Arg Cys
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Ser Met Asp Leu Lys Asn Ile Asn Phe
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<213> Homo sapiens

<400> 23

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            20                  25                  30

Gly Ala Gly Thr Cys Cys Ala Ile Ser Leu Trp Leu Arg Gly Leu Arg
        35                  40                  45
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Met Cys Thr Pro Leu Gly Arg Glu Gly Glu Glu Cys His Pro Gly Ser
50 55 60

His Lys Val Pro Phe Phe Arg Lys Arg Lys His His Thr Cys Pro Cys
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Ser Met Asp Leu Lys Asn Ile Asn Phe
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<212> DNA
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<211> 382
<212> DNA
<213> Homo sapiens

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gcacctgctg tgccatcagc ctgtggcttc gagggctgcg gatgtgcacc ccgctggggc 180
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accacacctg tccttgcttg cccaacctgc tgtgctccag gttcccggac ggcaggtacc 300
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<210> 31
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<222> (7)
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<210> 32
<211> 80
<212> PRT
<213> Unknown Organism

<220>
<223> Description of Unknown Organism: Mamba Instestinal Toxin 1 (MIT1)

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20 25 30

Pro Val Gly Thr Ser Gly Glu Asp Cys His Pro Ala Ser His Lys Ile
35 40 45

Pro Phe Ser Gly Gln Arg Met His His Thr Cys Pro Cys Ala Pro Asn
50 55 60

Leu Ala Cys Val Gln Thr Ser Pro Lys Lys Phe Lys Cys Leu Ser Lys
65 70 75 80

<210> 33
<211> 86
<212> PRT
<213> Homo sapiens

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            20                  25                  30

Pro Leu Gly Arg Glu Gly Glu Glu Cys His Pro Gly Ser His Lys Ile
        35                  40                  45

Pro Phe Phe Arg Lys Arg Lys His His Thr Cys Pro Cys Leu Pro Asn
    50                  55                  60

Leu Leu Cys Ser Arg Phe Pro Asp Gly Arg Tyr Arg Cys Ser Met Asp
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Leu Lys Asn Ile Asn Phe
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            20                  25                  30

Pro Leu Gly Arg Glu Gly Glu Glu Cys His Pro Gly Ser His Lys Val
        35                  40                  45

Pro Phe Phe Arg Lys Arg Lys His His Thr Cys Pro Cys Leu Pro Asn
    50                  55                  60

Leu Leu Cys Ser Arg Phe Pro Asp Gly Arg Tyr Arg Cys Ser Met Asp
65                  70                  75                  80

Leu Lys Asn Ile Asn Phe
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Attorney Docket No. 57127 (46342)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT: T. Watanabe, et al. **EXAMINER:** Not Yet Assigned

U.S.S.N.: Not Yet Assigned – based on PCT/JP00/05684 **GROUP:** Not Yet Assigned

FILED: February 27, 2002

FOR: NOVEL G PROTEIN-COUPLED RECEPTOR PROTEIN and DNA THEREOF

BOX PATENT APPLICATION
Commissioner for Patents
Washington, D.C. 20231

CERTIFICATE OF MAILING

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being deposited with the United States Postal Service, in an envelope with sufficient postage as Certified Express Mail No: EL933048924US addressed to : Box Patent Application, Commissioner for Patents, Washington, D.C. 20231 on February 27, 2002 .

By: _____
Fatima H. DeArruda

Sir:

PRELIMINARY AMENDMENT

Please preliminarily amend the subject application as follows:

IN THE SPECIFICATION

Please amend page 86, lines 28-29, as follows:

--heating at 94°C for 30 seconds followed by 68°C for 4 minutes.- -

Please amend page 87, lines 8-9, as follows:

--heating at 94°C for 30 seconds followed by 68°C for 4 minutes.--

Please amend page 87, lines 26-27, as follows:

--cycle of heating at 94°C for 30 seconds followed by 68°C for 4 minutes.--

REMARKS

The amendments to the specification are to correct obvious typographical errors and do not introduce new matter.

CONCLUSION

Applicants submit that all claims are allowable as written and respectfully request early favorable action by the Examiner. If the Examiner believes that a telephone conversation with Applicants' agent would expedite prosecution of this application, the Examiner is cordially invited to call the undersigned agent of record.

Respectfully submitted

Date: February 27, 2002

By: Dianne Rees

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Marked-Up Version of Sections of Specification Showing Changes Being Made

At page 86, lines 28-29:

--heating at 94°C for 30 seconds followed by 68°C for 4[4] minutes.--

At page 87, lines 8-9:

--heating at 94°C for 30 seconds followed by 68°C for 4[4] minutes.--

At page 87, lines 26-27:

--cycle of heating at 94°C for 30 seconds followed by 68°C for 4[4] minutes.- -

11/prts

SPECIFICATION

NOVEL G PROTEIN-COUPLED RECEPTOR PROTEIN AND DNA THEREOF

FIELD OF THE INVENTION

The present invention relates to a human brain-derived novel protein (G protein-coupled receptor protein) or its salt, a DNA encoding the same and the like.

BACKGROUND ART

A variety of physiologically active substances such as hormones, neurotransmitters, etc. regulate the functions in vivo through specific receptor proteins located in a cell membrane. Many of these receptor proteins are coupled with guanine nucleotide-binding protein (hereinafter sometimes referred to as G protein) and mediate the intracellular signal transduction via activation of G protein. These receptor proteins possess the common structure, i.e. seven transmembrane domains and are thus collectively referred to as G protein-coupled receptors or seven-transmembrane receptors.

G protein-coupled receptor proteins present on the cell surface of each functional cells and organs in the body, and play important physiological roles as the targets of molecules that regulate the functions of the cells and organs, e.g., hormones, neurotransmitters, physiologically active substances and the like.

To clarify the relationship between substances that regulate complex biological functions in various cells and organs and their specific receptor proteins, in particular, G protein-coupled receptor proteins, would elucidate the functional mechanisms in various cells and organs in the body to provide a very important means for development of drugs closely associated with the functions.

For example, in central nervous system organs such as brain, their physiological functions of brain are controlled in vivo through regulation by many hormones, hormone-like

substances, neurotransmitters or physiologically active substances. In particular, physiologically active substances are found in numerous sites of the brain and regulate the physiological functions through their corresponding receptor proteins. However, it is supposed that many unknown hormones, neurotransmitters or other physiologically active substances still exist in the brain and, as for their cDNAs encoding receptor proteins, many of such cDNAs have not yet been reported. In addition, it is still unknown if there are subtypes of known receptor proteins.

It is also very important for development of drugs to clarify the relationship between substances that regulate elaborate functions in brain and their specific receptor proteins. Furthermore, for efficient screening of agonists and antagonists to receptor proteins in development of drugs, it is required to clarify functional mechanisms of receptor protein genes expressed in brain and express the genes in an appropriate expression system.

In recent years, random analysis of cDNA sequences has been actively studied as a means for analyzing genes expressed in vivo. The sequences of cDNA fragments thus obtained have been registered on and published to databases as Expressed Sequence Tag (EST). However, since many ESTs comprise sequence information only, it is difficult to deduce their functions from the information.

DISCLOSURE OF THE INVENTION

The present invention provides a human brain-derived novel protein (G protein-coupled receptor protein), its partial peptide, or their salts, a DNA comprising a DNA encoding said protein or its partial peptide, a recombinant vector comprising said DNA, a transformant transformed by said vector, a process for producing said protein or its salt, an antibody against said protein, its partial peptide or their salts, determination of a ligand to the protein (G protein-coupled receptor protein), a method for screening a compound or its salt that alters the binding property

between a ligand and the protein or its salt, a kit for the screening described above, a compound or its salts that alters the binding property between a ligand and the protein (G protein-coupled receptor protein), which is obtained by the screening method or the screening kit, and a pharmaceutical composition comprising a compound or its salt that alters the binding property between a ligand and the protein (G protein-coupled receptor protein).

The present inventors have made extensive studies and as a result, succeeded in isolating cDNAs encoding a human brain-derived novel protein (G protein-coupled receptor protein) and in sequencing their full base sequences. When the base sequences were translated into the amino acid sequences, 1 to 7 transmembrane domains were found to be on the hydrophobic plot, verifying that the proteins encoded by these cDNAs are seven-transmembrane type G protein-coupled receptor proteins (Fig. 3). The present inventors have continued extensive studies and as a result, have come to accomplish the present invention.

Thus, the present invention provides, for example, the following:

(1) A protein which comprises the same or substantially the same amino acid sequence as the amino acid sequence represented by SEQ ID NO:1, or a salt thereof;

(2) A partial peptide of the protein according to the above (1), or a salt thereof;

(3) A DNA which comprises a DNA encoding the protein according to the above (1);

(4) A DNA according to the above (3) having a base sequence represented by SEQ ID NO:2 or SEQ ID NO:3;

(5) A recombinant vector which comprises the DNA according to the above (3);

(6) A transformant transformed with the recombinant vector according to the above (5);

(7) A method for producing the protein or a salt thereof according to the above (1), which comprises culturing said transformant according to the above (6), and producing and accumulating the protein according to the above (1);

(8) An antibody to the protein according to the above (1) or the partial peptide according to the above (2), or a salt thereof;

(9) A method for determination of a ligand to the protein or its salt according to the above (1), which comprises using the protein according to the above (1) or the partial peptide according to the above (2), or a salt thereof;

(10) A method for screening a compound or its salt that alters the binding property between a ligand and the protein or its salt according to the above (1), which comprises using the protein according to the above (1) or the partial peptide according to the above (2), or a salt thereof;

(11) A kit for screening a compound or its salt that alters the binding property between a ligand and the protein or its salt according to the above (1), whidh comprises the protein according to the above (1) or the partial peptide according to the above (2), or a salt thereof;

(12) A compound or its salt that alters the binding property between a ligand and the protein or its salt according to the above (1), which is obtainable by using the screening method according to the above (10) or the screening kit according to the above (11);

(13) A pharmaceutical composition which comprises a compound or its salt that alters the binding property between a ligand and the protein or its salt according to the above (1), which is obtainable by using the screening method according to the above (10) or the screening kit according to the above (11); and

(14) A DNA that hybridizes to the DNA according to the above (3) under highly stringent conditions.

More specifically, the present invention further provides, for example, the following:

(15) A protein or its salt according to the above (1), wherein the protein is a protein comprising (i) an amino acid sequence represented by SEQ ID NO:1 of which at least 1 or 2 (preferably 1 to 30, more preferably 1 to 9 and most

preferably several (1 or 2)) amino acids are deleted; (ii) an amino acid sequence represented by SEQ ID NO:1 to which at least 1 or 2 (preferably 1 to 30, more preferably 1 to 10 and most preferably several (1 or 2)) amino acids are added; (iii) an amino acid sequence represented by SEQ ID NO:1 into which at least 1 or 2 (preferably 1 to 30, more preferably 1 to 10 and most preferably several (1 or 2)) amino acids are substituted; or (iv) a combination of the above amino acid sequences;

(16) A method for determination of a ligand according to the above (10), which is by bringing a test compound in contact with the protein or a salt thereof, according to the above (1) or the partial peptide or a salt thereof, according to the above (2);

(17) A method for determination of a ligand according to the above (9) wherein the ligand is angiotensin, bombesin, canavinoid, cholecystokinin, glutamine, serotonin, melatonin, neuropeptide Y, opioid, purines, vasopressin, oxytocin, PACAP, secretin, glucagon, calcitonin, adrenomedulin, somatostatin, GHRH, CRF, ACTH, GRP, PTH, VIP (vasoactive intestinal polypeptide), somatostatin, dopamine, motilin, amylin, bradykinin, CGRP (calcitonin gene-related peptide), leukotrienes, pancreastatin, prostaglandins, thromboxane, adenosine, adrenaline, α and β-chemokines (e.g., IL-8, GROα, GROβ, GROγ, NAP-2, ENA-78, PF4, IP10, GCP-2, MCP-1, HC14, MCP-3, I-309, MIP1α, MIP-1β, RANTES, etc.), endothelin, enterogastrin, histamin, neurotensin, TRH, pancreatic polypeptide, galanin, Mamba Intestinal Toxin 1(may be referred as MIT1; Toxicon, 28 847-856, 1990 FEBS Letters 461, 183-188(1999)) or a homologue to the mamals;

(18) A method of screening according to the above (11), wherein (i) the case where the ligand is contacted with the protein or its salt according to the above (1) or the partial peptide or its salt according to the above (2) is compared with (ii) the case where the ligand and a test compound are contacted with the protein or its salt according to the above (1) or the partial peptide or its salt according to the above (2);

(19) A method of screening a compound or its salt that alters the binding property between a ligand and the protein or its salt according to the above (1), which comprises measuring the amounts of a labeled ligand bound to the protein or its salt according to the above (1) or to the partial peptide or its salt according to the above (2), (i) when the labeled ligand is brought in contact with the protein or its salt according to the above (1) or with the partial peptide or its salt according to the above (2), and (ii) when the labeled ligand and a test compound are brought in contact with the protein or its salt according to the above (1) or with the partial peptide or its salt according to the above (2); and comparing the amounts measured in (i) and (ii);

(20) A method of screening a compound or its salt that alters the binding property between a ligand and the protein or its salt according to the above (1), which comprises measuring the amounts of a labeled ligand bound to a cell comprising the protein according to the above (1), (i) when the labeled ligand is brought in contact with the cell comprising the protein according to the above (1), and (ii) when the labeled ligand and a test compound are brought in contact with the cell comprising the protein according to the above (1); and comparing the amounts measured in (i) and (ii);

(21) A method of screening a compound or its salt that alters the binding property between a ligand and the protein or its salt according to the above (1), which comprises measuring the amounts of a labeled ligand bound to a cell membrane fraction comprising the protein according to the above (1), (i) when the labeled ligand is brought in contact with the cell membrane fraction, and (ii) when the labeled ligand and a test compound are brought in contact with the cell membrane fraction; and comparing the amounts measured in (i) and (ii);

(22) A method of screening a compound or its salt that alters the binding property between a ligand and the protein or its salt according to the above (1), which comprises

measuring the amounts of a labeled ligand bound to a protein expressed in a cell membrane, (i) when the labeled ligand is brought in contact with the protein expressed in a cell membrane of the transformant according to the above (6) by culturing the transformant and (ii) when the labeled ligand and a test compound are brought in contact with the protein expressed in a cell membrane of the transformant according to the above (6) by culturing the transformant; and comparing the amounts measured in (i) and (ii);

(23) A method of screening a compound or its salt that alters the binding property between a ligand and the protein or its salt according to the above (1), which comprises measuring the protein-mediated cell stimulating activities, (i) when a compound that activates the protein or its salt according to the above (1) is brought in contact with a cell comprising the protein according to the above (1), and (ii) when a compound that activates the protein or its salt according to the above (1) and a test compound are brought in contact with a cell comprising the protein according to the above (1); and comparing the activities measured in (i) and (ii);

(24) A method of screening a compound or its salt that alters the binding property between a ligand and the protein or its salt according to the above (1), which comprises measuring the protein-mediated cell stimulating activities, when a compound that activates the protein or its salt according to the above (1) is brought in contact with a protein expressed in a cell membrane of the transformant according to the above (6) by culturing the transformant, and when the compound that activates the protein or its salt according to the above (1) and a test compound are brought in contact with the protein expressed in a cell membrane of the transformant according to the above (6) by culturing the transformant; and comparing the protein-mediated activities measured in (i) and (ii);

(25) A method of screening according to the above (23) or (24), wherein the compound whidh activates the protein according to the above (1) is angiotensin, bombesin,

canavinoid, cholecystokinin, glutamine, serotonin, melatonin, neuropeptide Y, an opioid, a purine, vasopressin, oxytocin, PACAP, secretin, glucagon, calcitnonin, adrenomedulin, somatostatin, GHRH, CRF, ACTH, GRP, PTH, vasoactive intestinal and related polypeptide (VIP), somatostatin, dopamine, motilin, amylin, bradykinin, calcitonin gene-related peptide (CGRP), a leukotriene, pancreastatin, a prostaglandin, thromboxane, adenosine, adrenaline, an α- and β-chemokine (e.g., IL-8, GROα, GROβ, GROγ, NAP-2, ENA-78, PF4, IP10, GCP-2, MCP-1, HC14, MCP-3, I-309, MIP1-α, MIP-1β, RANTES, etc.), endothelin, enterogastrin, histamine, neurotensin, TRH, pancreatic polypeptide, galanin, MIT1 or their homologue to the mammals;

(26) A compound or its salt that alters the binding property between a ligand and the protein or its salt according to the above (1), which is obtainable by using the screening method according to the above (18) to (25);

(27) A pharmaceutical composition which comprises a compound or its salt that alters the binding property between a ligand and the protein or its salt according to the above (1), which is obtainable by using the screening method according to the above (18) to (25);

(28) A kit for screening according to the above (11), which comprises the cell comprising the protein according to the above (1);

(29) A kit for screening according to the above (11), which comprises the cell membrane fraction comprising the protein according to the above (1);

(30) A kit for screening according to the above (11), which is characterized by comprising the protein expressed at the cell membrane of a transformatant by culturing the transformant according to the above (6);

(31) A compound or salts that alters the binding property between a ligand and the protein or its salt according to the above (1), which is obtainable by using the screening method according to the above (28)to (30);

(32) A pharmaceutical composition comprising a compound or a salts that alters the binding property between a ligand

and the protein or its salt according to the above (1), which is obtainable by using the screening method according to the above (28) to (30);

(33) A method of quantifying the protein according to the above (1), the partial peptide according to the above (2), or a salt thereof, which comprises contacting the antibody according to the above (8) with the protein according to the above (1), the partial peptide according to the above (2), or a salt thereof;

(34) A method of quantifying the protein according to the above (1), the partial peptide according to the above (2) or salts thereof in a test fluid, which comprises competitively reacting the antibody according to the above (8) with a test fluid and a labeled form of the protein according to the above (1), the partial peptide according to the above (2) or salts thereof; and measuring the ratios bound to the antibody of the labeled form of the protein according to the above (1), the partial peptide or its salts according to the above (2);

(35) A method of quantifying the protein according to the above (1), the partial peptide according to the above (2), or salts thereof in a test fluid, which comprises reacting a test fluid simultaneously or sequentially with the antibody according to the above (8) immobilized on a carrier and the labeled antibody according to the above (8), and then measuring the activity of the label on the immobilizing carrier, and so forth.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows the base sequence of DNA encoding the human brain-derived protein (ZAQC) of the present invention obtained in Example 1, and the amino acid sequence deduced from the base sequence (following to Figure 2).

Fig. 2 shows the base sequence of DNA encoding the human brain-derived protein (ZAQC) of the present invention obtained in Example 1, and the amino acid sequence deduced from the base sequence (continued from Figure 1 and following to Figure 3).

Fig. 3 shows the base sequence of DNA encoding the human brain-derived protein (ZAQC) of the present invention obtained in Example 1, and the amino acid sequence deduced from the base sequence (continued from Figure 2).

Fig. 4 shows the base sequence of DNA encoding the human brain-derived protein (ZAQT) of the present invention obtained in Example 1, and the amino acid sequence deduced from the base sequence (following to Figure 5).

Fig. 5 shows the base sequence of DNA encoding the human brain-derived protein (ZAQT) of the present invention obtained in Example 1, and the amino acid sequence deduced from the base sequence (continued from Figure 4 and following to Figure 6).

Fig. 6 shows the base sequence of DNA encoding the human brain-derived protein (ZAQT) of the present invention obtained in Example 1, and the amino acid sequence deduced from the base sequence (continued from Figure 5).

Fig. 7 shows the hydrophobic plotting of the human brain-derived protein of the present invention.

Fig. 8 shows the results of the analysis on the distribution of ZAQ expression, which was performed in Example 2.

Fig. 9 shows the amino acid sequence of MIT1, Human type ZAQ ligand precursor peptide (A type) and Human type ZAQ ligand precursor peptide (G type).

In the figure, "MIT1" represents the amino acid sequence of MIT1; "Human (A type)" represents the amino acid sequence of Human type ZAQ ligand maturation peptide (A type); and "Human (B type)" represents the amino acid sequence of Human type ZAQ ligand maturation peptide (B type).

Fig. 10 shows the results of the measurement for the ZAQ activating function of the purified ZAQ ligand peptide, which was performed in Example 6 (6-3).

Fig. 11 shows the restriction map of the plasmid pCAN618, which was used in Example 5 (5-1).

BEST MODE OF EMBODIMENT OF THE INVENTION

The protein (G protein-coupled receptor protein) of the present invention is the receptor protein which comprises the same or substantially the same amino acid sequence as the amino acid sequence [amino acid sequence in Fig. 1 to Fig. 3 or Fig. 4 to Fig. 6] shown by SEQ ID NO:1 (hereinafter the protein(G protein-coupled receptor protein) and its salt are sometimes referred to as the protein of the present invention).

The protein (G protein-coupled receptor protein) of the present invention may be any protein derived from any cells of human and other mammmals (e.g. guinea pig, rat, mouse, rabbit, swine, sheep, bovine, monkey, etc.) such as splenic cell, nerve cell, glial cell, β cell of pancreas, bone marrow cell, mesangial cell, Langerhans' cell, epidermic cell, epithelial cell, endothelial cell, fibroblast, fibrocyte, myocyte, fat cell, immune cell (e.g., macrophage, T cell, B cell, natural killer cell, mast cell, neutrophil, basophil, eosinophil, monocyte), megakaryocyte, synovial cell, chondrocyte, bone cell, osteoblast, osteoclast, mammary gland cell, hepatocyte, interstitial cell, etc., the corresponding precursor cells, stem cells, cancer cells and hemocyte type cells (e.g., MEL, M1, CTLL-2, HT-2, WEHI-3, HL-60, JOSK-1, K562, ML-1, MOLT-3, MOLT-4, MOLT-10, CCRF-CEM, TALL-1, Jurkat, CCRT-HSB-2, KE-37, SKW-3, HUT-78, HUT-102, H9, U937, THP-1, HEL, JK-1, CMK, KO-812, MEG-01, etc.); or any tissues where such cells are present, such as brain or any of brain regions (e.g., olfactory bulb, amygdaloid nucleus, cerebral basal bulb, hippocampus, thalamus, hypothalamus, substhanlamic nucleus, cerebral cortex, medulla oblongata, cerebellum, occipital pole, frontal lobe, temporal lobe, putamen, caudate nucleus, corpus callosum, substantia nigra), spinal cord, hypophysis, stomach, pancreas, kidney, liver, gonad, thyroid, gall-bladder, bone marrow, adrenal gland, skin, muscle, lung, gastrointestinal tract (e.g., large intestine and small intestine), blood vessel, heart, thymus, spleen, submandibular gland, peripheral blood, pheripheral hemocyte, prostate, testis, ovary, placenta, uterus, bone, joint, skeletal muscle,

(especially, brain and brain region) etc.; the proteins may also be synthetic proteins.

The amino acid sequence which has substantially the same amino acid sequence as that represented by SEQ ID NO:1 includes an amino acid sequence having at least about 90% homology, preferably at least about 95% homology, and more preferably at least about 98% homology, to the amino acid sequence represented by SEQ ID NO:1.

Preferred examples of the protein having substantially the same amino acid sequence as that represented by SEQ ID NO: 1 are proteins having substantially the same amino acid sequence as that represented by SEQ ID NO: 1 and having substantially the same activity as that of the amino acid sequence represented by SEQ ID NO: 1.

Preferred examples of the protein of the present invention, which comprises the same or substantially the same amino acid sequence as that represented by SEQ ID NO: 1 are proteins comprisising the same or substantially the same amino acid sequence as that represented by SEQ ID NO: 1 and having substantially the same activity as that of the amino acid sequence represented by SEQ ID NO: 1. The substantially equivalent activities are, for example, a ligand binding activity, a signal transduction activity, etc. The term "substantially equivalent" is used to mean that the nature of these activities is equivalent. Therefore, it is preferred that these activities such as ligand binding activity, a signal transduction activity, etc. are equivalent in strength (e.g., about 0.5 to about 2 times), and it is allowable that even differences among grades such as the strength of these activities and molecular weight of the porotein are present.

The activities such as a ligand binding activity, a signal transduction activity or the like can be assayed according to a publicly known method, for example, by means of ligand determination or screening, which will be later described.

The protein of the present invention which can be employed include proteins comprising (i) an amino acid

sequence represented by SEQ ID NO:1, of which at least 1 or 2 (preferably 1 to 30, more preferably 1 to 10 and most preferably several (1 or 2)) amino acids are deleted; (ii) an amino acid sequence represented by SEQ ID NO:1, to which at least 1 or 2 (preferably 1 to 30, more preferably 1 to 10 and most preferably several (1 or 2)) amino acids are added; (iii) an amino acid sequence represented by SEQ ID NO:1, in which at least 1 or 2 (preferably 1 to 30, more preferably 1 to 10 and most preferably several (1 or 2)) amino acids are substituted by other amino acids; and (iv) a combination of the above amino acid sequences.

Throughout the present specification, the proteins are represented in accordance with the conventional way of describing peptides, that is, the N-terminus (amino terminus) at the left hand and the C-terminus (carboxyl terminus) at the right hand. In the proteins of the present invention including the proteins comprising the amino acid sequence shown by SEQ ID NO:1, the C-terminus is usually in the form of a carboxyl group (-COOH) or a carboxylate (-COO$^-$) but may be in the form of an amide (-$CONH_2$) or an ester (-COOR).

Examples of the ester group shown by R include a C_{1-6} alkyl group such as methyl, ethyl, n-propyl, isopropyl, n-butyl, etc.; a C_{3-8} cycloalkyl group such as cyclopentyl, cyclohexyl, etc.; a C_{6-12} aryl group such as phenyl, α-naphthyl, etc.; an aralkyl having 7 to 14 carbon atoms such as a phenyl-C_{1-2} alkyl group, e.g., benzyl, phenethyl, etc.; a α-naphthyl-C_{1-2} alkyl group such as α-naphthylmethyl, etc.; and the like. In addition, pivaloyloxymethyl or the like which is used widely as an ester for oral administration may also be used.

Where the protein of the present invention comprises a carboxyl group (or a carboxylate) at a position other than the C-terminus, it may be amidated or esterified and such an amide or ester is also included within the protein of the present invention. The ester group may be the same group as that described with respect to the above C-terminal.

Furthermore, examples of the protein of the present invention include variants of the above protein, wherein the amino group at the N-terminus (e.g., methionine residue) of the peptide is protected with a protecting group (e.g., a C_{1-6} acyl group such as a C_{1-6} alkanoyl group, e.g., formyl group, acetyl group, etc.); those wherein the N-terminal region is cleaved in vivo and the glutamyl group thus formed is pyroglutaminated; those wherein a substituent (e.g., -OH, -SH, amino group, imidazole group, indole group, guanidino group, etc.) on the side chain of an amino acid in the molecule is protected with a suitable protecting group (e.g., a C_{1-6} acyl group such as a C_{2-6} alkanoyl group, e.g., formyl group, acetyl group, etc.), or conjugated proteins such as glycoproteins having sugar chains.

Specific examples of the protein of the present invention include a human-derived receptor (preferably human brain-derived) protein comprising the amino acid sequence represented by SEQ ID NO:1, etc.

As the partial peptide of protein of the present invention (hereinafter sometimes referred to as partial peptide), any partial peptide described for the protein can be used. For example, a part of the protein molecule of the present invention which is exposed to outside of a cell membrane or the like can be used so long as it has a receptor binding activity.

Specifically, the partial peptide of the protein of the present invention having the amino acid sequence represented by SEQ ID NO:1 (shown Fig. 7) is a peptide comprising the parts, which have been analyzed to be extracellular domains (hydrophilic domains) in the hydrophobic plotting analysis. A peptide comprising a hydrophobic domain part can be used as well. In addition, the peptide may comprise each domain separately or plural domains together.

The partial peptide of the present invention is a peptide having at least 20, preferably at least 50 and more preferably at least 100 amino acids, in the amino acid sequence, which constitutes the protein of the present invention.

The substantially the same amino acid sequence includes an amino acid sequence having at least about 50% homology, preferably at least about 70% homology, more preferably at least about 80% homology, much more preferably at least about 90% homology and most preferably at least about 95% homology, to the amino acid sequence represented.

As used herein the term "substantially equivalent activities" refers to the same significance as defined hereinabove. The "substantially equivalent activities" can be assayed by the same method as described above.

In the partial peptide of the present invention, at least 1 or 2 (preferably 1 to 10, more preferably several (1 or 2)) amino acids may be deleted; at least 1 or 2 (preferably 1 to 20, more preferably 1 to 10 and most preferably several (1 or 2)) amino acids may be added; or at least 1 or 2 (preferably 1 to 10, more preferably 1 to 5, furhter preferably several (1 or 2)), amino acids may be substituted by other amino acids.

In the partial peptide in the protein of the present invention, the C-terminus is usually in the form of a carboxyl group (-COOH) or a carboxylate ($-COO^-$) but may be in the form of an amide ($-CONH_2$) or an ester (-COOR), as in the protein of the present invention described above.

Furthermore, examples of the partial peptide of the present invention include variants of the above peptides, wherein the amino group at the N-terminal methionine residue is protected with a protecting group, those wherein the N-terminal region is cleaved in vivo and the Gln formed is pyroglutaminated, those wherein a substituent on the side chain of an amino acid in the molecule is protected with a suitable protecting group, or conjugated proteins such as glycoproteins having sugar chains, as in the protein of the present invention described above.

Moreover, in the partial peptide in the protein of the present invention, the C-terminus is usually in the form of a carboxyl group (-COOH) or a carboxylate ($-COO^-$) but may be in the form of an amide ($-CONH_2$) or an ester (-COOR), as in the protein of the present invention described above.

As the salts of the protein of the present invention or its partial peptide, physiologically acceptable acid addition salts are particularly preferred. Examples of such salts are salts with inorganic acids (e.g., hydrochloric acid, phosphoric acid, hydrobromic acid, sulfuric acid), salts with organic acids (e.g., acetic acid, formic acid, propionic acid, fumaric acid, maleic acid, succinic acid, tartaric acid, citric acid, malic acid, oxalic acid, benzoic acid, methanesulfonic acid, benzenesulfonic acid) and the like.

The protein of the present invention or salts thereof may be manufactured by a publicly known method used to purify a polypeptide from human or other warm-blooded animal cells or tissues described above. Alternatively, the protein of the present invention or salts thereof may also be manufactured by culturing a transformant comprising DNA encoding the protein of the present invention, as will be later described. Furthermore, the protein of the present invention or salts thereof may also be manufactured by the methods for synthesizing proteins, which will also be described hereinafter, or by modified methods.

Where the protein or salts thereof are manufactured from human or mammalian tissues or cells, human or mammalian tissues or cells are homogenized, then extracted with an acid or the like, and the extract is isolated and purified by a combination of chromatography techniques such as reverse phase chromatography, ion exchange chromatography, and the like.

To synthesize the protein of the present invention, its partial peptide or its salts or amides, commercially available resins that are used for protein synthesis may be used. Examples of such resins include chloromethyl resin, hydroxymethyl resin, benzhydrylamine resin, aminomethyl resin, 4-benzyloxybenzyl alcohol resin, 4-methylbenzhydrylamine resin, PAM resin, 4-hydroxymethylmehtylphenyl acetamidomethyl resin, polyacrylamide resin, 4-(2′,4′-dimethoxyphenyl-hydroxymethyl)phenoxy resin, 4-(2′,4′-dimethoxyphenyl-Fmoc-

aminoethyl) phenoxy resin, etc. Using these resins, amino acids in which α-amino groups and functional groups on the side chains are appropriately protected are condensed on the resin in the order of the sequence of the objective protein according to various condensation methods publicly known in the art. At the end of the reaction, the protein is excised from the resin and at the same time, the protecting groups are removed. Then, intramolecular disulfide bond-forming reaction is performed in a highly diluted solution to obtain the objective protein or amides thereof.

For condensation of the protected amino acids described above, a variety of activation reagents for protein synthesis may be used, but carbodiimides are particularly preferably employed. Examples of such carbodiimides include DCC, N,N'-diisopropylcarbodiimide, N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide, etc. For activation by these reagents, the protected amino acids in combination with a racemization inhibitor (e.g., HOBt, HOOBt) are added directly to the resin, or the protected amino acids are previously activated in the form of symmetric acid anhydrides, HOBt esters or HOOBt esters, followed by adding the thus activated protected amino acids to the resin.

Solvents suitable for use to activate the protected amino acids or condense with the resin may be chosen from solvents that are known to be usable for protein condensation reactions. Examples of such solvents are acid amides such as N,N-dimethylformamide, N,N-dimethylacetamide, N-methylpyrrolidone, etc.; halogenated hydrocarbons such as methylene chloride, chloroform, etc.; alcohols such as trifluoroethanol, etc.; sulfoxides such as dimethylsulfoxide, etc.; ethers such as pyridine, dioxane, tetrahydrofuran, etc.; nitriles such as acetonitrile, propionitrile, etc.; esters such as methyl acetate, ethyl acetate, etc.; and appropriate mixtures of these solvents. The reaction temperature is appropriately chosen from the range known to be applicable to protein binding reactions and is usually selected in the range of approximately -20°C to 50°C. The activated amino acid derivatives are used generally in an

excess of 1.5 to 4 times. The condensation is examined using the ninhydrin reaction; when the condensation is insufficient, the condensation can be completed by repeating the condensation reaction without removal of the protecting groups. When the condensation is yet insufficient even after repeating the reaction, unreacted amino acids are acetylated with acetic anhydride or acetylimidazole to cancel any possible adverse affect on the subsequent reaction.

Examples of the protecting groups used to protect the starting amino groups include Z, Boc, t-pentyloxycarbonyl, isobornyloxycarbonyl, 4-methoxybenzyloxycarbonyl, Cl-Z, Br-Z, adamantyloxycarbonyl, trifluoroacetyl, phthaloyl, formyl, 2-nitrophenylsulphenyl, diphenylphosphinothioyl, Fmoc, etc.

A carboxyl group can be protected by, e.g., alkyl esterification (in the form of linear, branched or cyclic alkyl esters of the alkyl moiety such as methyl, ethyl, propyl, butyl, t-butyl, cyclopentyl, cyclohexyl, cycloheptyl, cyclooctyl, 2-adamantyl, etc.), aralkyl esterification (e.g., esterification in the form of benzyl ester, 4-nitrobenzyl ester, 4-methoxybenzyl ester, 4-chlorobenzyl ester, benzhydryl ester, etc.), phenacyl esterification, benzyloxycarbonyl hydrazidation, t-butoxycarbonyl hydrazidation, trityl hydrazidation, or the like.

The hydroxyl group of serine can be protected through, for example, its esterification or etherification. Examples of groups appropriately used for the esterification include a lower alkanoyl group, such as acetyl group, an aroyl group such as benzoyl group, and a group derived from carbonic acid such as benzyloxycarbonyl group and ethoxycarbonyl group. Examples of a group appropriately used for the etherification include benzyl group, tetrahydropyranyl group, t-butyl group, etc.

Examples of groups for protecting the phenolic hydroxyl group of tyrosine include Bzl, Cl_2-Bzl, 2-nitrobenzyl, Br-Z, t-butyl, etc.

Examples of groups used to protect the imidazole moiety of histidine include Tos, 4-methoxy-2,3,6-

trimethylbenzenesulfonyl, DNP, benzyloxymethyl, Bum, Boc, Trt, Fmoc, etc.

Examples of the activated carboxyl groups in the starting amino acids include the corresponding acid anhydrides, azides, activated esters (esters with alcohols (e.g., pentachlorophenol, 2,4,5-trichlorophenol, 2,4-dinitrophenol, cyanomethyl alcohol, p-nitrophenol, HONB, N-hydroxysuccimide, N-hydroxyphthalimide, HOBt)). As the activated amino acids in which the amino groups are activated in the starting material, the corresponding phosphoric amides are employed.

To eliminate (split off) the protecting groups, there are used catalytic reduction under hydrogen gas flow in the presence of a catalyst such as Pd-black or Pd-carbon; an acid treatment with anhydrous hydrogen fluoride, methanesulfonic acid, trifluoromethanesulfonic acid or trifluoroacetate, or a mixture solution of these acids; a treatment with a base such as diisopropylethylamine, triethylamine, piperidine or piperazine; and reduction with sodium in liquid ammonia. The elimination of the protecting group by the acid treatment described above is carried out generally at a temperature of approximately -20°C to 40°C. In the acid treatment, it is efficient to add a cation scavenger such as anisole, phenol, thioanisole, m-cresol, p-cresol, dimethylsulfide, 1,4-butanedithiol or 1,2-ethanedithiol. Furthermore, 2,4-dinitrophenyl group known as the protecting group for the imidazole of histidine is removed by a treatment with thiophenol. Formyl group used as the protecting group of the indole of tryptophan is eliminated by the aforesaid acid treatment in the presence of 1,2-ethanedithiol or 1,4-butanedithiol, as well as by a treatment with an alkali such as a dilute sodium hydroxide solution and dilute ammonia.

Protection of functional groups that should not be involved in the reaction of the starting materials, protecting groups, elimination of the protecting groups and activation of functional groups involved in the reaction may

be appropriately selected from publicly known groups and publicly known means.

In another method for obtaining the amides of the protein of the present invention, for example, the α-carboxyl group of the carboxyl terminal amino acid is first protected by amidation; the peptide (protein) chain is then extended from the amino group side to a desired length. Thereafter, a protein in which only the protecting group of the N-terminal α-amino group has been eliminated from the peptide and a protein in which only the protecting group of the C-terminal carboxyl group has been eliminated are manufactured. The two proteins are condensed in a mixture of the solvents described above. The details of the condensation reaction are the same as described above. After the protected protein obtained by the condensation is purified, all the protecting groups are eliminated by the method described above to give the desired crude protein. This crude protein is purified by various known purification means. Lyophilization of the major fraction gives the amide of the desired protein.

To prepare the esterified protein of the present invention, for example, the α-carboxyl group of the carboxyl terminal amino acid is condensed with a desired alcohol to prepare the amino acid ester, which is followed by procedure similar to the preparation of the amidated protein above to give the desired esterified protein.

The partial peptide or salts of the protein of the present invention can be manufactured by publicly known methods for peptide synthesis, or by cleaving the protein of the present invention with an appropriate peptidase. For the methods for peptide synthesis, for example, either solid phase synthesis or liquid phase synthesis may be used. That is, the partial peptide or amino acids that can construct the protein of the present invention are condensed with the remaining part of the partial peptide of the present invention. Where the product comprises protecting groups, these protecting groups are removed to give the desired peptide. Publicly known methods for condensation and

elimination of the protecting groups are described in 1) - 5) below.

1) M. Bodanszky & M.A. Ondetti: Peptide Synthesis, Interscience Publishers, New York (1966)

2) Schroeder & Luebke: The Peptide, Academic Press, New York (1965)

3) Nobuo Izumiya, et al.: *Peptide Gosei-no-Kiso to Jikken* (Basics and experiments of peptide synthesis), published by Maruzen Co. (1975)

4) Haruaki Yajima & Shunpei Sakakibara: *Seikagaku Jikken Koza* (Biochemical Experiment) 1, *Tanpakushitsu no Kagaku* (Chemistry of Proteins) IV, 205 (1977)

5) Haruaki Yajima ed.: *Zoku Iyakuhin no Kaihatsu* (A sequel to Development of Pharmaceuticals), Vol. 14, Peptide Synthesis, published by Hirokawa Shoten

After completion of the reaction, the product may be purified and isolated by a combination of conventional purification methods such as solvent extraction, distillation, column chromatography, liquid chromatography and recrystallization to give the partial peptide of the present invention. When the partial peptide obtained by the above methods is in a free form, the peptide can be converted into an appropriate salt by a publicly known method; when the protein is obtained in a salt form, it can be converted into a free form or a different salt form by a publicly known method.

The DNA encoding the protein of the present invention may be any DNA so long as it comprises the base sequence encoding the protein of the present invention described above. Such a DNA may also be any one of genomic DNA, genomic DNA library, cDNA derived from the cells or tissues described above, cDNA library derived from the cells or tissues described above and synthetic DNA.

The vector to be used for the library may be any of bacteriophage, plasmid, cosmid, phagemid and the like. In addition, the DNA can be amplified by reverse transcriptase

polymerase chain reaction (hereinafter abbreviated as RT-PCR) with total RNA or mRNA fraction prepared from the above-described cells or tissues.

Specifically, the DNA encoding the protein of the present invention may be any one of, for example, DNA having the base sequence represented by SEQ ID NO:2 or SEQ ID NO:3, or any DNA having a base sequence hybridizable to the base sequence represented by SEQ ID NO:2 or SEQ ID NO:3 under high stringent conditions and encoding a protein which has the activities substantially equivalent to those of the protein of the present invention (e.g., a ligand binding activity, a signal transduction activity, etc.).

Specific examples of the DNA that is hybridizable to the base sequence represented by SEQ ID NO:2 or SEQ ID NO:3 under high stringent conditions include DNA having at least about 90% homology, preferably at least about 95% homology and more preferably at least about 98% homology, to the base sequence represented by SEQ ID NO:2 or SEQ ID NO:3.

The hybridization can be carried out by publicly known methods or by a modification thereof, for example, according to the method described in Molecular Cloning, 2nd Ed., J. Sambrook et al., Cold Spring Harbor Lab. Press, (1989). A commercially available library may also be used according to the instructions of the attached manufacturer's protocol. The hybridization can be carried out preferably under high stringent conditions.

The high stringent conditions used herein are, for example, those in a sodium concentration at about 19 mM to about 40 mM, preferably about 19 mM to about 20 mM at a temperature of about 50°C to about 70°C, preferably about 60°C to about 65°C. In particular, hybridization conditions in a sodium concentration at about 19 mM at a temperature of about 65°C are most preferred.

More specifically, for the DNA encoding the protein having the amino acid sequence represented by SEQ ID NO:1, there may be employed DNA having the base sequence represented by SEQ ID NO:2 or SEQ ID NO:3.

The nucleotides (oligonucleotide) comprising the base sequence encoding the protein of the present invention or a part of the base sequence complementary to the DNA is used to mean that not only the DNA encoding the partial peptide of the present invention described below but also RNA are embraced.

According to the present invention, antisense nucleotides (oligonucleotides) that can inhibit replication or expression of the protein of the resent invention can be designed and synthesized based on the cloned or determined base sequence information of the DNA encoding the protein. Such a (oligo) nucleotide (nucleic acid) is capable of hybridizing with RNA of G protein coupled protein gene to inhibit the synthesis or function of said RNA or capable of modulating the expression of a G protein-coupled receptor protein gene via interaction with G protein coupled protein-associated RNA. (oligo) nucleotides complementary to selected sequences of RNA associated with G protein-coupled receptor protein and (oligo) nucleotides specifically hybridizable with the selected sequences of RNA associated with G protein-coupled protein are useful in modulating or controlling the expression of a G protein coupled protein gene in vivo and in vitro, and in treating or diagnosing disease later described. The term "corresponding" is used to mean homologous to or complementary to a particular sequence of the base sequence or nucleic acid including the gene. The term "corresponding" between nucleotides, base sequences or nucleic acids and peptides (proteins) usually refers to amino acids of a peptide (protein) under the order derived from the sequence of nucleotides (nucleic acids) or their complements. 5' end hairpin loop, 5' end 6-base-pair repeats, 5' end untranslated region, polypeptide translation initiation codon, protein coding region, ORF translation initiation codon, 3' untranslated region, 3' end palindrome region, and 3' end hairpin loop in the G protein-coupled protein gene may be selected as preferred target regions, though any other region may be selected as a target in G protein coupled protein genes.

The relationship between the targeted nucleic acids and the (oligo) nucleotides complementary to at least a part of the target, specifically the relationship between the target and the (oligo) nucleotides hybridizable to the target, can be denoted to be "antisense". Examples of the antisense (oligo) nucleotides include polydeoxynucleotides comprising 2-deoxy-D-ribose, polydeoxynucleotides comprising D-ribose, any other type of polynucleotides which are N-glycosides of a purine or pyrimidine base, or other polymers comprising non-nucleotide backbones (e.g., protein nucleic acids and synthetic sequence-specific nucleic acid polymers commercially available) or other polymers comprising nonstandard linkages (provided that the polymers comprise nucleotides having such a configuration that allows base pairing or base stacking, as is found in DNA or RNA), etc. The antisense polynucleotides may be double-stranded DNA, single-stranded DNA, single-stranded RNA or a DNA:RNA hybrid, and may further include unmodified polynucleotides (or unmodified oligonucleotides), those with publicly known types of modifications, for example, those with labels known in the art, those with caps, methylated polynucleotides, those with substitution of one or more naturally occurring nucleotides by their analogue, those with intramolecular modifications of nucleotides such as those with uncharged linkages (e.g., methyl phosphonates, phosphotriesters, phosphoramidates, carbamates, etc.) and those with charged linkages or sulfur-comprising linkages (e.g., phosphorothioates, phosphorodithioates, etc.), those having side chain groups such as proteins (nucleases, nuclease inhibitors, toxins, antibodies, signal peptides, poly-L-lysine, etc.), saccharides (e.g., monosaccharides, etc.), those with intercalators (e.g., acridine, psoralen, etc.), those comprising chelators (e.g., metals, radioactive metals, boron, oxidative metals, etc.), those comprising alkylating agents, those with modified linkages (e.g., α anomeric nucleic acids, etc.), and the like. Herein the terms "nucleoside", "nucleotide" and "nucleic acid" are used to refer to moieties that comprise not only the purine and

pyrimidine bases, but also other heterocyclic bases, which have been modified. Such modifications may include methylated purines and pyrimidines, acylated purines and pyrimidines and other heterocyclic rings. Modified nucleotides and modified nucleotides also include modifications on the sugar moiety, wherein, for example, one or more hydroxyl groups may optionally be substituted with a halogen atom(s), an aliphatic group(s), etc., or may be converted into the corresponding functional groups such as ethers, amines, or the like.

The antisense polynucleotide (nucleic acid) of the present invention is RNA, DNA or a modified nucleic acid (RNA, DNA). Specific examples of the modified nucleic acid are, but not limited to, sulfur and thiophosphate derivatives of nucleic acids and those resistant to degradation of polynucleoside amides or oligonucleoside amides. The antisense nucleic acids of the present invention can be modified preferably based on the following design, that is, by increasing the intracellular stability of the antisense nucleic acid, increasing the cellular permeability of the antisense nucleic acid, increasing the affinity of the nucleic acid to the targeted sense strand to a higher level, or minimizing the toxicity, if any, of the antisense nucleic acid.

Many of such modifications are known in the art, as disclosed in J. Kawakami, et al., Pharm. Tech. Japan, Vol. 8, pp. 247, 1992; Vol. 8, pp. 395, 1992; S. T. Crooke, et al. ed., Antisense Research and Applications, CRC Press, 1993; etc.

The antisense nucleic acid of the present invention may comprise altered or modified sugars, bases or linkages. The antisense nucleic acid may also be provided in a specialized form such as liposomes, microspheres, or may be applied to gene therapy, or may be provided in combination with attached moieties. Such attached moieties include polycations such as polylysine that act as charge neutralizers of the phosphate backbone, or hydrophobic moieties such as lipids (e.g., phospholipids, cholesterols,

etc.) that enhance the interaction with cell membranes or increase uptake of the nucleic acid. Preferred examples of the lipids to be attached are cholesterols or derivatives thereof (e.g., cholesteryl chloroformate, cholic acid, etc.). These moieties may be attached to the nucleic acid at the 3' or 5' ends thereof and may also be attached thereto through a base, sugar, or intramolecular nucleoside linkage. Other moieties may be capping groups specifically placed at the 3' or 5' ends of the nucleic acid to prevent degradation by nucleases such as exonuclease, RNase, etc. Such capping groups include, but are not limited to, hydroxyl protecting groups known in the art, including glycols such as polyethylene glycol, tetraethylene glycol and the like.

The inhibitory action of the antisense nucleic acid can be examined using the transformant of the present invention, the gene expression system of the present invention in vivo and in vitro, or the translation system of the G protein-coupled receptor protein in vivo and in vitro. The nucleic acid can be applied to cells by a variety of publicly known methods.

The DNA encoding the partial peptide of the present invention may be any DNA so long as it comprises the base sequence encoding the partial peptide of the present invention described above. The DNA may also be any of genomic DNA, genomic DNA library, cDNA derived from the cells and tissues described above, cDNA library derived from the cells and tissues described above and synthetic DNA. The vector to be used for the library may be any of bacteriophage, plasmid, cosmid and phagemid. The DNA may also be directly amplified by reverse transcriptase polymerase chain reaction (hereinafter abbreviated as RT-PCR) using mRNA fraction prepared from the cells and tissues described above.

Specifically, the DNA encoding the partial peptide of the present invention may be any one of, for example, (1) DNA comprising a partial base sequence of the DNA having the base sequence represented by SEQ ID NO:2 or or SEQ ID NO:3, or (2) any DNA comprising a partial base sequence of the DNA

having a base sequence hybridizable to the base sequence represented by SEQ ID NO:2 or SEQ ID NO:3 under highly stringent conditions and encoding a protein which has the activities (e.g., a ligand-biding activity, a signal transduction activity, etc.) substantially equivalent to those of the protein peptide of the present invention.

Specific examples of the DNA that is hybridizable to the base sequence represented by SEQ ID NO:2 or SEQ ID NO:3 include DNA comprising a base sequence having at least about 90% homology, preferably at least about 95% homology and morepreferably at least about 98% homology, to the base sequence represented by SEQ ID NO:2 or SEQ ID NO:3.

For cloning of the DNA that completely encodes the protein of the present invention or its partial peptide (hereinafter sometimes collectively referred to as the protein of the present invention), the DNA may be either amplified by PCR using synthetic DNA primers comprising a part of the base sequence of the protein of the present invention, or the DNA inserted into an appropriate vector can be selected by hybridization with a labeled DNA fragment or synthetic DNA that encodes a part or entire region of the protein of the present invention. The hybridization can be carried out, for example, according to the method described in Molecular Cloning, 2nd, J. Sambrook et al., Cold Spring Harbor Lab. Press, 1989. The hybridization may also be performed using commercially available library in accordance with the protocol described in the attached instructions.

Conversion of the base sequence of the DNA can be effected by publicly known methods such as the Gupped duplex method or the Kunkel method or its modification by using a publicly known kit available as Mutan™-G or Mutan™-K (both manufactured by Takara Shuzo Co., Ltd.).

The cloned DNA encoding the protein can be used as it is, depending upon purpose or, if desired, after digestion with a restriction enzyme or after addition of a linker thereto. The DNA may comprise ATG as a translation initiation codon at the 5' end thereof and may further comprise TAA, TGA or TAG as a translation termination codon at the 3' end thereof.

These translation initiation and termination codons may also be added by using an appropriate synthetic DNA adapter.

The expression vector for the protein of the present invention can be manufactured, for example, by (a) excising the desired DNA fragment from the DNA encoding the protein of the present invention, and then (b) ligating the DNA fragment with an appropriate expression vector downstream a promoter in the vector.

Examples of the vector include plasmids derived form E. coli (e.g., pBR322, pBR325, pUC12, pUC13), plasmids derived from Bacillus subtilis (e.g., pUB110, pTP5, pC194), plasmids derived from yeast (e.g., pSH19, pSH15), bacteriophages such as λ phage, etc., animal viruses such as retrovirus, vaccinia virus, baculovirus, etc. as well as pA1-11, pXT1, pRc/CMV, pRc/RSV, pcDNAI/Neo, pcDNA3.1, pRc/CMV2, pRc/RSV (Invitrogen), etc.

The promoter used in the present invention may be any promoter if it matches well with a host to be used for gene expression. In the case of using animal cells as the host, examples of the promoter include SRα promoter, SV40 promoter, HIV-LTR promoter, CMV promoter, HSV-TK promoter, etc.

Among them, CMV promoter or SRα promoter is preferably used. Where the host is bacteria of the genus Escherichia, preferred examples of the promoter include trp promoter, lac promoter, recA promoter, λP_L promoter, lpp promoter, etc. In the case of using bacteria of the genus Bacillus as the host, preferred example of the promoter are SPO1 promoter, SPO2 promoter and penP promoter. When yeast is used as the host, preferred examples of the promoter are PHO5 promoter, PGK promoter, GAP promoter and ADH promoter. When insect cells are used as the host, preferred examples of the promoter include polyhedrin prompter and P10 promoter.

In addition to the foregoing examples, the expression vector may further optionally comprise an enhancer, a splicing signal, a poly A addition signal, a selection marker, SV40 replication origin (hereinafter sometimes abbreviated as SV40ori) etc. Examples of the selection marker include dihydrofolate reductase (hereinafter

sometimes abbreviated as dhfr) gene [methotrexate (MTX) resistance], ampicillin resistant gene (hereinafter sometimes abbreviated as Amp^r), neomycin resistant gene (hereinafter sometimes abbreviated as Neo^r, G418 resistance), etc. In particular, when dhfr gene is used as the selection marker in CHO (dhfr$^-$) cells, selection can also be made on thymidine free media.

If necessary and desired, a signal sequence that matches with a host is added to the N-terminus of the protein of the present invention. Examples of the signal sequence that can be used are Pho A signal sequence, OmpA signal sequence, etc. in the case of using bacteria of the genus Escherichia as the host; α-amylase signal sequence, subtilisin signal sequence, etc. in the case of using bacteria of the genus Bacillus as the host; MFα signal sequence, SUC2 signal sequence, etc. in the case of using yeast as the host; and insulin signal sequence, α-interferon signal sequence, antibody molecule signal sequence, etc. in the case of using animal cells as the host, respectively.

Using the vector comprising the DNA encoding the protein of the present invention thus constructed, transformants can be manufactured.

Examples of the host, which may be employed, are bacteria belonging to the genus Escherichia, bacteria belonging to the genus Bacillus, yeast, insect cells, insects and animal cells, etc.

Specific examples of the bacteria belonging to the genus Escherichia include Escherichia coli K12 DH1 (Proc. Natl. Acad. Sci. U.S.A., 60, 160 (1968)), JM103 (Nucleic Acids Research, 9, 309 (1981)), JA221 (Journal of Molecular Biology, 120, 517 (1978)), HB101 (Journal of Molecular Biology, 41, 459 (1969)), C600 (Genetics, 39, 440 (1954)), etc.

Examples of the bacteria belonging to the genus Bacillus include Bacillus subtilis MI114 (Gene, 24, 255 (1983)), 207-21 (Journal of Biochemistry, 95, 87 (1984)), etc.

Examples of yeast include Saccharomyces cereviseae AH22, AH22R⁻, NA87-11A, DKD-5D, 20B-12, Schizosaccharomyces pombe NCYC1913, NCYC2036, Pichia pastoris, etc.

Examples of insect cells include, for the virus AcNPV, Spodoptera frugiperda cells (Sf cells), MG1 cells derived from mid-intestine of Trichoplusia ni, High Five™ cells derived from egg of Trichoplusia ni, cells derived from Mamestra brassicae, cells derived from Estigmena acrea, etc.; and for the virus BmNPV, Bombyx mori N cells (BmN cells), etc. are used. Examples of the Sf cell which can be used are Sf9 cells (ATCC CRL1711) and Sf21 cells (both cells are described in Vaughn, J. L. et al., In Vivo, 13, 213-217 (1977).

As the insect, for example, a larva of Bombyx mori can be used (Maeda, et al., Nature, 315, 592 (1985)).

Examples of animal cells include monkey cells COS-7, Vero, Chinese hamster cells CHO (hereinafter referred to as CHO cells), dhfr gene deficient Chinese hamster cells CHO (hereinafter simply referred to as CHO(dhfr⁻) cell), mouse L cells, mouse AtT-20, mouse myeloma cells, rat GH3, human FL cells, etc.

Bacteria belonging to the genus Escherichia can be transformed, for example, by the method described in Proc. Natl. Acad. Sci. U.S.A., 69, 2110 (1972) or Gene, 17, 107 (1982). Bacteria belonging to the genus Bacillus can be transformed, for example, by the method described in Molecular & General Genetics, 168, 111 (1979).

Yeast can be transformed, for example, by the method described in Methods in Enzymology, 194, 182-187 (1991), Proc. Natl. Acad. Sci. U.S.A., 75, 1929 (1978), etc.

Insect cells or insects can be transformed, for example, according to the method described in Bio/Technology, 6, 47-55(1988), etc.

Animal cells can be transformed, for example, according to the method described in *Saibo Kogaku* (Cell Engineering), extra issue 8, *Shin Saibo Kogaku Jikken Protocol* (New Cell Engineering Experimental Protocol), 263-267 (1995), published by Shujunsha, or Virology, 52, 456 (1973).

Thus, the transformant transformed with the expression vector comprising the DNA encoding the G protein-coupled receptor protein can be obtained.

Where the host is bacteria belonging to the genus Escherichia or the genus Bacillus, the transformant can be appropriately incubated in a liquid medium which comprises materials required for growth of the transformant such as carbon sources, nitrogen sources, inorganic materials, and so on. Examples of the carbon sources include glucose, dextrin, soluble starch, sucrose, etc. Examples of the nitrogen sources include inorganic or organic materials such as ammonium salts, nitrate salts, corn steep liquor, peptone, casein, meat extract, soybean cake, potato extract, etc. Examples of the inorganic materials are calcium chloride, sodium dihydrogenphosphate, magnesium chloride, etc. In addition, yeast, vitamins, growth promoting factors etc. may also be added to the medium. Preferably, pH of the medium is adjusted to about 5 to about 8.

A preferred example of the medium for incubation of the bacteria belonging to the genus Escherichia is M9 medium supplemented with glucose and Casamino acids (Miller, Journal of Experiments in Molecular Genetics, 431-433, Cold Spring Harbor Laboratory, New York, 1972). If necessary and desired, a chemical such as 3β-indolylacrylic acid can be added to the medium thereby to activate the promoter efficiently. Where the bacteria belonging to the genus Escherichia are used as the host, the transformant is usually cultivated at about 15°C to about 43°C for about 3 hours to about 24 hours. If necessary and desired, the culture may be aerated or agitated.

Where the bacteria belonging to the genus Bacillus are used as the host, the transformant is cultivated generally at about 30°C to about 40°C for about 6 hours to about 24 hours. If necessary and desired, the culture can be aerated or agitated.

Where yeast is used as the host, the transformant is cultivated, for example, in Burkholder's minimal medium (Bostian, K. L. et al., Proc. Natl. Acad. Sci. U.S.A., 77,

4505 (1980)) or in SD medium supplemented with 0.5% Casamino acids (Bitter, G. A. et al., Proc. Natl. Acad. Sci. U.S.A., 81, 5330 (1984)). Preferably, pH of the medium is adjusted to about 5 to about 8. In general, the transformant is cultivated at about 20°C to about 35°C for about 24 hours to about 72 hours. If necessary and desired, the culture can be aerated or agitated.

Where insect cells or insects are used as the host, the transformant is cultivated in, for example, Grace's Insect Medium (Grace, T. C. C., Nature, 195, 788 (1962)) to which an appropriate additive such as immobilized 10% bovine serum is added. Preferably, pH of the medium is adjusted to about 6.2 to about 6.4. Normally, the transformant is cultivated at about 27°C for about 3 days to about 5 days and, if necessary and desired, the culture can be aerated or agitated.

Where animal cells are employed as the host, the transformant is cultivated in, for example, MEM medium comprising about 5% to about 20% fetal bovine serum (Science, 122, 501 (1952)), DMEM medium (Virology, 8, 396 (1959)), RPMI 1640 medium (The Journal of the American Medical Association, 199, 519 (1967)), 199 medium (Proceeding of the Society for the Biological Medicine, 73, 1 (1950)), etc. Preferably, pH of the medium is adjusted to about 6 to about 8. The transformant is usually cultivated at about 30°C to about 40°C for about 15 hours to about 60 hours and, if necessary and desired, the culture can be aerated or agitated.

As described above, the G protein-coupled receptor protein of the present invention can be produced in the cell membrane of the transformant, etc.

The protein of the present invention can be separated and purified from the culture described above by the following procedures.

When the protein of the present invention is extracted from the culture or cells, after cultivation the transformants or cells are collected by a publicly known method and suspended in an appropriate buffer. The

transformants or cells are then disrupted by publicly known methods such as ultrasonication, a treatment with lysozyme and/or freeze-thaw cycling, followed by centrifugation, filtration, etc. Thus, the crude extract of the protein of the present invention can be obtained. The buffer used for the procedures may comprise a protein modifier such as urea or guanidine hydrochloride, or a surfactant such as Triton X-100™, etc. When the protein is secreted in the culture, after completion of the cultivation the supernatant can be separated from the transformants or cells to collect the supernatant by a publicly known method.

The protein comprised in the supernatant or the extract thus obtained can be purified by appropriately combining the publicly known methods for separation and purification. Such publicly known methods for separation and purification include a method utilizing difference in solubility such as salting out, solvent precipitation, etc.; a method utilizing mainly difference in molecular weight such as dialysis, ultrafiltration, gel filtration, SDS-polyacrylamide gel electrophoresis, etc.; a method utilizing difference in electric charge such as ion exchange chromatography, etc.; a method utilizing difference in specific affinity such as affinity chromatography, etc.; a method utilizing difference in hydrophobicity such as reverse phase high performance liquid chromatography, etc.; a method utilizing difference in isoelectric point such as isoelectrofocusing electrophoresis; and the like.

When the protein thus obtained is in a free form, it can be converted into the salt by publicly known methods or modifications thereof. On the other hand, when the protein is obtained in the form of a salt, it can be converted into the free form or in the form of a different salt by publicly known methods or modifications thereof.

The protein produced by the recombinant can be treated, prior to or after the purification, with an appropriate protein modifying enzyme so that the protein can be appropriately modified to partially remove a polypeptide. Examples of the protein-modifying enzyme include trypsin,

chymotrypsin, arginyl endopeptidase, protein kinase, glycosidase or the like.

The activity of the thus produced protein of the present invention or salts thereof can be determined by a test binding to a labeled ligand, by an enzyme immunoassay using a specific antibody, or the like.

Antibodies to the protein of the present invention, its partial peptides, or salts thereof may be any of polyclonal antibodies and monoclonal antibodies, as long as they are capable of recognizing the protein of the present invention, its partial peptides, or salts thereof.

The antibodies to the protein of the present invention, its partial peptides, or salts thereof (hereinafter sometimes merely referred to as the protein of the present invention) may be manufactured by publicly known methods for manufacturing antibodies or antisera, using as antigens the protein of the present invention.

[Preparation of monoclonal antibody]

(a) Preparation of monoclonal antibody-producing cells

The polypeptide or protein of the present invention is administered to warm-blooded animals either solely or together with carriers or diluents to the site where the production of antibody is possible by the administration. In order to potentiate the antibody productivity upon the administration, complete Freund's adjuvants or incomplete Freund's adjuvants may be administered. The administration is usually carried out once every two to six weeks and two to ten times in total. Examples of the applicable warm-blooded animals are monkeys, rabbits, dogs, guinea pigs, mice, rats, sheep and goats, with the use of mice and rats being preferred.

In the preparation of monoclonal antibody-producing cells, a warm-blooded animal, e.g., mice, immunized with an antigen wherein the antibody titer is noted is selected, then spleen or lymph node is collected after two to five days from the final immunization and antibody-producing cells comprised therein are fused with myeloma cells from

homozoic or heterozoic animal to give monoclonal antibody-producing hybridomas. Measurement of the antibody titer in antisera may be carried out, for example, by reacting a labeled polypeptide, which will be described later, with the antiserum followed by assaying the binding activity of the labeling agent bound to the antibody. The fusion may be carried out, for example, by the known method by Koehler and Milstein (Nature, 256, 495, 1975). Examples of the fusion accelerator are polyethylene glycol (PEG), Sendai virus, etc., of which PEG is preferably employed.

Examples of the myeloma cells are those collected from warm-blooded animals such as NS-1, P3U1, SP2/0, etc. In particular, P3U1 is preferably employed. A preferred ratio of the count of the antibody-producing cells used (spleen cells) to the count of myeloma cells is within a range of approximately 1:1 to 20:1. When PEG (preferably, PEG 1000 to PEG 6000) is added in a concentration of approximately 10 to 80% followed by incubating at 20 to 40° C, preferably at 30 to 37° C for 1 to 10 minutes, an efficient cell fusion can be carried out.

Various methods can be used for screening of a monoclonal antibody-producing hybridoma. Examples of such methods include a method which comprises adding the supernatant of hybridoma to a solid phase (e.g., microplate) adsorbed with the polypeptide (protein) as an antigen directly or together with a carrier, adding an anti-immunoglobulin antibody (where mouse cells are used for the cell fusion, anti-mouse immunoglobulin antibody is used) labeled with a radioactive substance or an enzyme or Protein A and detecting the monoclonal antibody bound to the solid phase, and a method which comprises adding the supernatant of hybridoma to a solid phase adsorbed with an anti-immunoglobulin antibody or Protein A, adding the polypeptide labeled with a radioactive substance or an enzyme and detecting the monoclonal antibody bound to the solid phase.

The monoclonal antibody can be selected according to publicly known methods or their modifications. In general, the selection can be effected in a medium for animal cells

supplemented with HAT (hypoxanthine, aminopterin and thymidine). Any selection and growth medium can be employed as far as the hybridoma can grow there. For example, RPMI 1640 medium comprising 1% to 20%, preferably 10% to 20% fetal bovine serum, GIT medium (Wako Pure Chemical Industries, Ltd.) comprising 1% to 10% fetal bovine serum, a serum free medium for cultivation of a hybridoma (SFM-101, Nissui Seiyaku Co., Ltd.) and the like can be used for the selection and growth medium. The cultivation is carried out generally at 20°C to 40°C, preferably at 37°C, for about 5 days to about 3 weeks, preferably 1 to 2 weeks, normally in 5% CO_2. The antibody titer of the culture supernatant of a hybridoma can be determined as in the assay for the antibody titer in antisera described above.

(b) Purification of monoclonal antibody

Separation and purification of a monoclonal antibody can be carried out by publicly known methods, such as separation and purification of immunoglobulins (for example, salting-out, alcohol precipitation, isoelectric point precipitation, electrophoresis, adsorption and desorption with ion exchangers (e.g., DEAE), ultracentrifugation, gel filtration, or a specific purification method which comprises collecting only an antibody with an activated adsorbent such as an antigen-binding solid phase, Protein A or Protein G and dissociating the binding to obtain the antibody.

[Preparation of polyclonal antibody]

The polyclonal antibody of the present invention can be manufactured by publicly known methods or modifications thereof. For example, a warm-blooded animal is immunized with an immunogen (protein antigen) per se, or a complex of immunogen and a carrier protein is formed and a warm-blooded animal is immunized with the complex in a manner similar to the method described above for the manufacture of monoclonal antibodies. The product comprising the antibody to the polypeptide of the present invention is collected from the immunized animal followed by separation and purification of the antibody.

In the complex of immunogen and carrier protein used to immunize a warm-blooded animal, the type of carrier protein and the mixing ratio of carrier to hapten may be any type and in any ratio, as long as the antibody is efficiently produced to the hapten immunized by crosslinking to the carrier. For example, bovine serum albumin, bovine thyroglobulin or hemocyanin is coupled to hapten in a carrier-to-hapten weight ratio of approximately 0.1 to 20, preferably about 1 to about 5.

A variety of condensation agents can be used for the coupling of carrier to hapten. Glutaraldehyde, carbodiimide, maleimide activated ester and activated ester reagents comprising thiol group or dithiopyridyl group are used for the coupling.

The condensation product is administered to warm-blooded animals either solely or together with carriers or diluents to the site that can produce the antibody by the administration. In order to potentiate the antibody productivity upon the administration, complete Freund's adjuvant or incomplete Freund's adjuvant may be administered. The administration is usually made once every 2 to 6 weeks and 3 to 10 times in total.

The polyclonal antibody can be collected from the blood, ascites, etc., preferably from the blood of warm-blooded animal immunized by the method described above.

The polyclonal antibody titer in antiserum can be assayed by the same procedure as that for the determination of serum antibody titer described above. The separation and purification of the polyclonal antibody can be carried out, following the method for the separation and purification of immunoglobulins performed as in the separation and purification of monoclonal antibodies described hereinabove.

The protein of the present invention, its partial peptides, or salts thereof and the DNA encoding the same can be used for; ① determination of ligands to the protein of the present invention; ② preparation of antibodies and antisera; ③ construction of recombinant protein expression systems; ④ development of the receptor binding assay

systems using the expression systems and screening of pharmaceutical candidate compounds; ⑤ effecting drug design based on comparison with structurally similar ligand receptors; ⑥ reagents for preparation of probes and PCR primers for gene diagnosis; ⑦ production of transgenic animals; and ⑧ pharmaceutical drugs for the gene prophylaxis and/or gene therapy.

In particular, by the use of the receptor binding assay system using the expression system of the recombinant G protein-coupled receptor protein of the present invention, compounds (e.g., agonists, antagonists, etc.) that alter the binding property of human- or mammal-specific ligands for the G protein-coupled receptor protein can be screened, and the agonists or antagonists can be used as prophylactic and/or therapeutic agents for various diseases.

Hereinafter, the protein of the present invention, its partial peptides, or salts thereof (hereinafter sometimes referred to as the protein of the present invention), the DNA encoding the protein of the present invention or its partial peptides (hereinafter sometimes referred to as the DNA of the present invention) and the antibodies to the protein of the present invention (hereinafter sometimes referred to as the antibodies of the present invention) are specifically described for the use or applications.

(1) Determination of a ligand (agonist) to the protein of the present invention

The protein or its salt of the present invention, or the partial peptide or its salts of the present invention are useful as reagents for searching and determining ligands (agonists) to the protein of the present invention or its salts.

That is, the present invention provides a method for determining a ligand to the protein of the present invention, which comprises bringing the protein of the present invention or its salts, or the partial peptide of the present invention or its salts, in contact with a test compound.

Examples of the test compound include publicly known ligands (e.g., angiotensin, bombesin, canavinoid, cholecystokinin, glutamine, serotonin, melatonin, neuropeptide Y, opioid, purines, vasopressin, oxytocin, PACAP, secretin, glucagon, calcitonin, adrenomedulin, somatostatin, GHRH, CRF, ACTH, GRP, PTH, VIP (vasoactive intestinal and related polypeptide), somatostatin, dopamine, motilin, amylin, bradykinin, CGRP (calcitonin gene-related peptide), leukotrienes, pancreastatin, prostaglandins, thromboxane, adenosine, adrenaline, α and β-chemokines (e.g., IL-8, GROα, GROβ, GROγ, NAP-2, ENA-78, PF4, IP10, GCP-2, MCP-1, HC14, MCP-3, I-309, MIP-1α, MIP-1β, RANTES, etc.), endothelin, enterogastrin, histamine, neurotensin, TRH, pancreatic polypeptide, galanin, MITI, their homologues to mammals, etc.) as well as other substances, for example, tissue extracts and cell culture supernatants from human and mammals (e.g., mice, rats, swine, bovine, sheep, monkeys, etc.). For example, the tissue extract or cell culture supernatant is added to the protein of the present invention and fractionated while assaying the cell stimulating activities, etc. to finally give a single ligand.

In the case where the ligands are peptidic lignds, such ligands are sometimes referred to as ligand peptides. Further, in the case where the ligand peptide is expressed as a precursor, and converted to a mature form by removal of a signal peptide, the precursor may be sometimes referred to as a ligand precursor peptide, and the mature form may be sometimes referred to as a ligand mature peptide, respectively. Both of the peptides may be merely abbreviated as the ligand peptides.

In more detail, the method for determining ligands of the present invention comprises determining compounds (e.g., peptides, proteins, non-peptide compounds, synthetic compounds, fermentation products, etc.) or salts thereof that bind to the protein of the present invention to provide cell stimulating activities (e.g., the activities that promote or suppress arachidonic acid release, acetylcholine release, intracellular Ca^{2+} release, intracellular cAMP

production, intracellular cGMP production, inositol phosphate production, change in cell membrane potential, phosphorylation of intracellular proteins, activation of c-fos, pH reduction, etc.), using the protein of the present invention, its partial peptides or salts thereof, or by the receptor binding assay using the constructed recombinant protein expression system.

The method for determining ligands of the present invention is characterized, for example, by measurement of the amount of the test compound bound to the protein or the partial peptide, or by assaying the cell-stimulating activities, etc., when the test compound is brought in contact with the protein of the present invention or its partial peptides.

More specifically, the present invention provides the following features:

(1) a method for determining a ligand to the protein of the present invention or its salt, which comprises bringing a labeled test compound in contact with the protein of the present invention or its salt or the partial peptide of the present invention or its salt and measuring the amount of the labeled test compound bound to the protein or its salt or to the partial peptide or its salt;

(2) a method for determining ligands to the protein of the present invention or its salt, which comprises bringing a labeled test compound in contact with cells or cell membrane fraction comprising the protein of the present invention, and measuring the amount of the labeled test compound bound to the cells or the membrane fraction;

(3) a method for determining ligands to the protein of the present invention, which comprises culturing a transformant comprising the DNA encoding the protein of the present invention, bringing a labeled test compound in contact with the receptor protein expressed on the cell membrane by said culturing, and measuring the amount of the labeled test compound bound to the protein or its salt;

(4) a method for determining ligands to the protein of the present invention or its salt, which comprises bringing a

test compound in contact with cells comprising the protein of the present invention and measuring the protein-mediated cell stimulating activities (e.g., the activities that promote or suppress arachidonic acid release, acetylcholine release, intracellular Ca^{2+} release, intracellular cAMP production, intracellular cGMP production, inositol phosphate production, change in cell membrane potential, phosphorylation of intracellular proteins, activation of c-fos, pH reduction, etc.); and,

(5) a method for determining ligands to the protein of the present invention or its salt, which comprises culturing a transformant comprising DNA encoding the protein of the present invention, bringing a labeled test compound in contact with the protein expressed on the cell membrane by said culturing, and measuring the protein-mediated cell stimulating activities (e.g., the activities that promote or suppress arachidonic acid release, acetylcholine release, intracellular Ca^{2+} release, intracellular cAMP production, intracellular cGMP production, inositol phosphate production, change in cell membrane potential, phosphorylation of intracellular proteins, activation of c-fos, pH reduction, etc.).

It is particularly preferred to perform the tests (1) to (3) described above, thereby to confirm that the test compound can bind to the protein of the present invention, followed by the tests (4) and (5) described above.

Any protein exemplified to be usable as the receptor protein for determining ligands, so long as it comprises the protein of the present invention or the partial peptide of the present invention. However, the protein that is abundantly expressed using animal cells is appropriate. The protein of the present invention can be manufactured by the method for expression described above, preferably by expressing DNA encoding the protein in mammalian or insect cells. As DNA fragments encoding the desired portion of the protein, complementary DNA is generally used but not necessarily limited thereto. For example, gene fragments or synthetic DNA may also be used. For introducing a DNA

fragment encoding the protein of the present invention into host animal cells and efficiently expressing the same, it is preferred to insert the DNA fragment downstream a polyhedrin promoter of nuclear polyhedrosis virus (NPV), which is a baculovirus having insect hosts, an SV40-derived promoter, a retrovirus promoter, a metallothionein promoter, a human heat shock promoter, a cytomegalovirus promoter, an SRα promoter or the like. The amount and quality of the receptor expressed can be determined by a publicly known method. For example, this determination can be made by the method described in the literature (Nambi, P., et al., J. Biol. Chem., 267, 19555-19559 (1992)).

Accordingly, the subject comprising the protein of the present invention, its partial peptides or salts thereof in the method for determining the ligand according to the present invention may be the protein, its partial peptides or salts thereof purified by publicly known methods, cells comprising the protein, or membrane fractions of such cells.

Where cells comprising the protein of the present invention are used in the method of the present invention for determination of ligands, the cells may be fixed using glutaraldehyde, formalin, etc. The fixation can be made by a publicly known method.

The cells comprising the protein of the present invention are host cells that have expressed the protein of the present invention, which host cells include Escherichia coli, Bacillus subtilis, yeast, insect cells, animal cells, and the like.

The cell membrane fraction refers to a fraction abundant in cell membrane obtained by cell disruption and subsequent fractionation by a publicly known method. Useful cell disruption methods include cell squashing using a Potter-Elvehjem homogenizer, disruption using a Waring blender or Polytron (manufactured by Kinematica Inc.), disruption by ultrasonication, and disruption by cell spraying through thin nozzles under an increased pressure using a French press or the like. Cell membrane fractionation is effected mainly by fractionation using a centrifugal force, such as

centrifugation for fractionation and density gradient centrifugation. For example, cell disruption fluid is centrifuged at a low speed (500 rpm to 3,000 rpm) for a short period of time (normally about 1 to about 10 minutes), the resulting supernatant is then centrifuged at a higher speed (15,000 rpm to 30,000 rpm) normally for 30 minutes to 2 hours. The precipitate thus obtained is used as the membrane fraction. The membrane fraction is rich in the protein expressed and membrane components such as cell-derived phospholipids and membrane proteins.

The amount of the protein in the cells comprising the protein and in the membrane fraction is preferably 10^3 to 10^8 molecules per cell, more preferably 10^5 to 10^7 molecules per cell. As the amount of expression increases, the ligand binding activity per unit of membrane fraction (specific activity) increases so that not only the highly sensitive screening system can be constructed but also large quantities of samples can be assayed with the same lot. To perform the methods (1) through (3) supra for determination of a ligand to the protein of the present invention or its salt, an appropriate protein fraction and a labeled test compound are required.

The protein fraction is preferably a fraction of naturally occurring receptor protein or a recombinant receptor fraction having an activity equivalent to that of the natural protein. Herein, the term "equivalent activity" is intended to mean a ligand binding activity, a signal transduction activity or the like that is equivalent to that possessed by naturally occurring receptor proteins. Preferred examples of labeled test compounds include angiotensin, bombesin, canavinoid, cholecystokinin, glutamine, serotonin, melatonin, neuropeptide Y, opioid, purines, vasopressin, oxytocin, PACAP, secretin, glucagon, calcitonin, adrenomedulin, somatostatin, GHRH, CRF, ACTH, GRP, PTH, VIP (vasoactive intestinal polypeptide), somatostatin, dopamine, motilin, amylin, bradykinin, CGRP (calcitonin gene-related peptide), leukotrienes, pancreastatin, prostaglandins, thromboxane, adenosine,

adrenaline, α and β-chemokines (e.g., IL-8, GROα, GROβ, GROγ, NAP-2, ENA-78, PF4, IP10, GCP-2, MCP-1, HC14, MCP-3, I-309, MIP1α, MIP-1β, RANTES, etc.), endothelin, enterogastrin, histamin, neurotensin, TRH, pancreatic polypeptide, galanin, MITI, or their homologue to mammals, etc.), which are labeled with [^{3}H], [^{125}I], [^{14}C], [^{35}S], etc.

More specifically, the ligand to the protein of the present invention or its salt is determined by the following procedures. First, a standard receptor preparation is prepared by suspending cells comprising the protein of the present invention or the membrane fraction thereof in a buffer appropriate for use in the determination method. Any buffer can be used so long as it does not inhibit the ligand-receptor binding, such buffers including a phosphate buffer or a Tris-HCl buffer having pH of 4 to 10 (preferably pH of 6 to 8). For the purpose of minimizing non-specific binding, a surfactant such as CHAPS, Tween-80™ (manufactured by Kao-Atlas Inc.), digitonin or deoxycholate, and various proteins such as bovine serum albumin or gelatin, may optionally be added to the buffer. Further for the purpose of suppressing the degradation of the receptors or ligands by proteases, a protease inhibitor such as PMSF, leupeptin, E-64 (manufactured by Peptide Institute, Inc.) and pepstatin may also be added. A given amount (5,000 to 500,000 cpm) of the test compound labeled with [^{3}H], [^{125}I], [^{14}C], [^{35}S] or the like is added to 0.01 ml to 10 ml of the receptor solution. To determine the amount of non-specific binding (NSB), a reaction tube comprising an unlabeled test compound in a large excess is also prepared. The reaction is carried out at approximately 0 to 50°C, preferably about 4 to 37°C for about 20 minutes to about 24 hours, preferably about 30 minutes to about 3 hours. After completion of the reaction, the reaction mixture is filtrated through glass fiber filter paper, etc. and washed with an appropriate volume of the same buffer. The residual radioactivity on the glass fiber filter paper is then measured by means of a liquid scintillation counter or γ-counter. A test compound exceeding 0 cpm in count obtained by subtracting nonspecific

binding (NSB) from the total binding (B) (B minus NSB) may be selected as a ligand (agonist) to the protein of the present invention or its salt.

The method (4) or (5) above for determination of a ligand to the protein of the present invention or its salt can be performed as follows. The protein-mediated cell-stimulating activities (e.g., the activities that promote or suppress arachidonic acid release, acetylcholine release, intracellular Ca^{2+} release, intracellular cAMP production, intracellular cGMP production, inositol phosphate production, change in cell membrane potential, phosphorylation of intracellular proteins, activation of c-fos, pH reduction, etc.) may be determined by a publicly known method, or using an assay kit commercially available. Specifically, cells comprising the protein are first cultured on a multi-well plate, etc. Prior to the ligand determination, the medium is replaced with fresh medium or with an appropriate non-cytotoxic buffer, followed by incubation for a given period of time in the presence of a test compound, etc. Subsequently, the cells are extracted or the supernatant is recovered and the resulting product is quantified by appropriate procedures. Where it is difficult to detect the production of the index substance (e.g., arachidonic acid) for the cell-stimulating activity due to a degrading enzyme compriseed in the cells, an inhibitor against such a degrading enzyme may be added prior to the assay. For detecting activities such as the cAMP production suppression activity, the baseline production in the cells is increased by forskolin or the like and the suppressing effect on the increased baseline production may then be detected.

The kit of the present invention for determination of the ligand that binds to the protein or its salt of the present invention comprises the protein or its salt of the present invention, the partial peptide or its salt of the present invention, cells comprising the protein of the present invention, or the membrane fraction of the cells comprising the protein of the present invention.

Examples of the ligand determination kit of the present invention are given below.

1. Reagents for determining ligands

(1) Buffers for assay and washing

Hanks' Balanced Salt Solution (manufactured by Gibco Co.) supplemented with 0.05% bovine serum albumin (Sigma Co.).

The solution is sterilized by filtration through a 0.45 μm filter and stored at 4°C. Alternatively, the solution may be prepared at use.

(2) Standard G protein-coupled receptor protein

CHO cells on which the protein of the present invention has been expressed are passaged in a 12-well plate in a density of 5×10^5 cells/well followed by culturing at 37°C under 5% CO_2 and 95% air for 2 days.

(3) Labeled test compounds

Compounds labeled with [^{3}H], [^{125}I], [^{14}C], [^{35}S], etc., which are commercially available labels, or compounds labeled by appropriate methods.

An aqueous solution of the compound is stored at 4°C or -20°C. The solution is diluted to 1 μM with an assay buffer at use. A sparingly water-soluble test compound is dissolved in dimethylformamide, DMSO, methanol, etc.

(4) Non-labeled compounds

A non-labeled form of the same compound as the labeled compound is prepared in a concentration 100 to 1,000-fold higher than that of the labeled compound.

2. Method for assay

(1) CHO cells expressing the protein of the present invention are cultured in a 12-well culture plate. After washing twice with 1 ml of an assay buffer, 490 μl of the assay buffer is added to each well.

(2) After 5 μl of the labeled test compound is added, the resulting mixture is reacted at room temperature for an hour. To determine the non-specific binding, 5 μl of the non-labeled compound is added to the system.

(3) The reaction mixture is removed and the wells are washed 3 times with 1 ml of washing buffer. The labeled test compound bound to the cells is dissolved in 0.2N NaOH-1% SDS and then mixed with 4 ml of liquid scintillator A (manufactured by Wako Pure Chemical Industries, Ltd.).

(4) The radioactivity is measured using a liquid scintillation counter (manufactured by Beckman Co.).

The ligands that bind to the protein of the present invention or its salt include substances specifically present in the brain, pituitary gland and pancreas. Examples of such ligands are angiotensin, bombesin, canavinoid, cholecystokinin, glutamine, serotonin, melatonin, neuropeptide Y, opioids, purines, vasopressin, oxytocin, PACAP, secretin, glucagon, calcitonin, adrenomedulin, somatostatin, GHRH, CRF, ACTH, GRP, PTH, VIP (vasoactive intestinal peptide), somatostatin, dopamine, motilin, amylin, bradykinin, CGRP (calcitonin gene-related peptide), leukotriens, pancreastatin, prostaglandins, thromboxane, adenosine, adrenaline, α and β-chemokines (e.g., IL-8, GROα, GROβ, GROγ, NAP-2, ENA-78, PF4, IP10, GCP-2, MCP-1, HC14, MCP-3, I-309, MIP1α, MIP-1β, RANTES, etc.), endothelin, enterogastrin, histamine, neurotensin, TRH, pancreatic polypeptide, galanin, MITI, their homologues to mammals, etc.

(2) Prophylactic and/or therapeutic agents for diseases associated with dysfunction of the G protein-coupled receptor protein of the present invention

When a ligand of the protein of the present invention is found by the methods described in (1), ① the protein of the present invention, or ② the DNA encoding the protein can be used, depending on the activities possessed by the ligand, as a prophylactic and/or therapeutic agent for diseases associated with dysfunction of the protein of the present invention.

For example, when the physiological activity of the ligand cannot be expected in a patient (deficiency of the protein) due to a decrease in the protein of the present invention, the activity of the ligand can be exhibited by:

① administering the protein of the present invention to the patient thereby to supplement the amount of the protein; or ② by increasing the amount of the protein in the patient through: i) administration of the DNA encoding the protein of the present invention to express the same in the patient; or ii) insertion and expression of the DNA encoding the protein of the present invention in the objective cells to transplant the cells to the patient, whereby the activity of the ligand can be sufficiently exhibited. That is, the DNA encoding the protein of the present invention is useful as a safe and low toxic prophylactic and/or therapeutic agent for diseases associated with dysfunction of the protein of the present invention.

The protein of the present invention and the DNA encoding the protein of the present invention are useful for the prevention and/or treatment of central dysfunction (e.g., Alzheimer's disease, senile dementia, suppression of eating (anorexia), epilepsy, etc.), hormone diseases (e.g., weak pains, atonic bleeding, before and after expulsion, subinvolution of uterus, cesarean section, induced abortion, galactostasis, etc.), liver/gallbladder/pancreas/endocrine-associated diseases (e.g., diabetes mellitus, suppression of eating, etc.), inflammatory diseases (e.g., allergy, asthma, rheumatoid, etc.), circulatory diseases (e.g., hypertension, cardiac hypertrophy, angina pectoris, arteriosclerosis, etc.), respiratory system diseases (e.g., pneumonia, asthma, bronchitis, respiratory system infectious diseases, chronical and ooclusive lung disease, etc.), and infectious diseases (e.g., ichorrhemia, MRSA, respiratory system infectious disease, urinary tract infectious disease, biliary infectious disease, infetciositive enrteritis, tympanitis, prostatitis, etc.).

The protein of the present invention and the DNA encoding the protein are particularly useful for the prevention and/or treatment of digestive system diseases (e.g., enrteritis, diarrhea, coprostasis, malabsorption syndrome, etc.)

When the protein of the present invention is used as the prophylactic/therapeutic agents supra, the protein can be prepared into a pharmaceutical composition in a conventional manner.

On the other hand, where the DNA encoding the protein of the present invention (hereinafter sometimes referred to as the DNA of the present invention) is used as the prophylactic/therapeutic agents described above, the DNA itself is administered; alternatively, the DNA is inserted into an appropriate vector such as retrovirus vector, adenovirus vector, adenovirus-associated virus vector, etc. and then administered in a conventional manner. The DNA of the present invention may also be administered as naked DNA, or with adjuvants to assist its uptake by gene gun or through a catheter such as a catheter with a hydrogel.

For example, ① the protein of the present invention or ② the DNA encoding the protein can be used orally, for example, in the form of tablets which may be sugar coated if necessary and desired, capsules, elixirs, microcapsules etc., or parenterally in the form of injectable preparations such as a sterile solution and a suspension in water or with other pharmaceutically acceptable liquid. These preparations can be manufactured by mixing ① the protein of the present invention or ② the DNA encoding the protein with a physiologically acceptable known carrier, a flavoring agent, an excipient, a vehicle, an antiseptic agent, a stabilizer, a binder, etc. in a unit dosage form required in a generally accepted manner that is applied to making pharmaceutical preparations. The effective component in the preparation is controlled in such a dose that an appropriate dose is obtained within the specified range given.

Additives miscible with tablets, capsules, etc. include a binder such as gelatin, corn starch, tragacanth and gum arabic, an excipient such as crystalline cellulose, a swelling agent such as corn starch, gelatin and alginic acid, a lubricant such as magnesium stearate, a sweetening agent such as sucrose, lactose and saccharin, and a flavoring agent such as peppermint, akamono oil and cherry. When the

unit dosage is in the form of capsules, liquid carriers such as oils and fats may further be used together with the additives described above. A sterile composition for injection may be formulated by conventional procedures used to make pharmaceutical compositions, e.g., by dissolving or suspending the active ingredients in a vehicle such as water for injection with a naturally occurring vegetable oil such as sesame oil and coconut oil, etc. to prepare the pharmaceutical composition. Examples of an aqueous medium for injection include physiological saline and an isotonic solution comprising glucose and other auxiliary agents (e.g., D-sorbitol, D-mannitol, sodium chloride, etc.) and may be used in combination with an appropriate dissolution aid such as an alcohol (e.g., ethanol or the like), a polyalcohol (e.g., propylene glycol and polyethylene glycol), a nonionic surfactant (e.g., polysorbate 80™ and HCO-50), etc. Examples of the oily medium include sesame oil and soybean oil, which may also be used in combination with a dissolution aid such as benzyl benzoate and benzyl alcohol.

The prophylactic/therapeutic agent described above may further be formulated with a buffer (e.g., phosphate buffer, sodium acetate buffer, etc.), a soothing agent (e.g., benzalkonium chloride, procaine hydrochloride, etc.), a stabilizer (e.g., human serum albumin, polyethylene glycol, etc.), a preservative (e.g., benzyl alcohol, phenol, etc.), an antioxidant, etc. The thus-prepared liquid for injection is normally filled in an appropriate ampoule.Since the thus obtained pharmaceutical preparation is safe and low toxic, the preparation can be administered to human or mammal (e.g., rats, rabbits, sheep, swine, bovine, cats, dogs, monkeys, etc.).

The dose of the protein or DNA of the present invention varies depending on subject to be administered, organs to be administered, conditions, routes for administration, etc.; in oral administration, e.g., for the adult patient suffering from a digestive disease, the dose is normally about 0.1 mg to about 100 mg, preferably about 1.0 to about 50 mg, and more preferably about 1.0 to about 20 mg per day

(as 60 kg body weight). In parenteral administration, the single dose varies depending on subject to be administered, target organ, conditions, routes for administration, etc. but it is advantageous, e.g., for the adult patient suffering from a digestive disease, to administer the active ingredient intravenously in a daily dose of about 0.01 to about 30 mg, preferably about 0.1 to about 20 mg, and more preferably about 0.1 to about 10 mg (as 60 kg body weight). For other animal species, the corresponding dose as converted per 60 kg body weight can be administered.

(3) Gene diagnostic agent

By using the DNA of the present invention as a probe, an abnormality (gene abnormality) of the DNA or mRNA encoding the protein of the present invention or its partial peptide in human or mammal (e.g., rats, rabbits, sheep, swine, bovine, cats, dogs, monkeys, etc.) can be detected. Therefore, the DNA of the present invention is useful as a gene diagnostic agent for the damage against the DNA or mRNA, its mutation, or its decreased expression, or increased expression or overexpression of the DNA or mRNA.

The gene diagnosis described above using the DNA of the present invention can be performed by, for example, the publicly known Northern hybridization assay or the PCR-SSCP assay (Genomics, 5, 874-879 (1989); Proceedings of the National Academy of Sciences of the United States of America, 86, 2766-2770 (1989)).

(4) Methods of quantifying ligands for protein of the present invention

Since the protein of the present invention has binding affinity to ligands, the ligand concentration can be quantified in vivo with good sensitivity.

The quantification methods of the present invention can be used in combination with, for example, a competitive method. The ligand concentration in a test sample can be measured by contacting the test sample to the protein of the present invention. Specifically, the methods can be used by

following, for example, the methods described in ① and ② below or its modified methods.

① Hiroshi Irie, ed. "Radioimmunoassay," Kodansha, published in 1974

② Hiroshi Irie, ed. "Sequel to the Radioimmunoassay," Kodansha, published in 1979

(5) Methods of screening compounds that alter the binding property between the protein of the present invention and ligands

Using the protein of the present invention, or using the receptor binding assay system of the expression system constructed using the recombinant protein, compounds (e.g., peptides, proteins, non-peptide compounds, synthetic compounds, fermentation products, etc.) or salt forms thereof that alter the binding property between ligands and the protein of the present invention can be efficiently screened.

Such compounds include (a) compounds that have the G protein-coupled receptor-mediated cell-stimulating activities (e.g., activities that promote or suppress arachidonic acid release, acetylcholine release, intracellular Ca^{2+} release, intracellular cAMP production, intracellular cGMP production, inositol phosphate production, changes in cell membrane potential, phosphorylation of intracellular proteins, activation of c-fos, pH reduction, etc.) (so-called agonists to the protein of the present invention); (b) compounds that do not have the cell-stimulating activity (so-called antagonists to the protein of the present invention); (c) compounds that potentiate the binding affinity between ligands and the protein of the present invention; and (d) compounds that reduce the binding affinity between ligands and the protein of the present invention (it is preferred to screen the compounds described in (a) using the ligand determination methods described above).

That is, the present invention provides methods of screening compounds or their salt forms that alter the

binding property between ligands and the protein, its partial peptide or salts thereof, which comprises comparing (i) the case wherein the protein of the present invention, its partial peptide or salts thereof are brought in contact with a ligand, with (ii) the case wherein the protein of the present invention, its partial peptide or salts thereof are brought in contact with a ligand and a test compound.

The screening methods of the present invention are characterized by assaying, for example, the amount of ligand bound to the protein, the cell-stimulating activity, etc., and comparing the property between (i) and (ii).

More specifically, the present invention provides the following screening methods:

① a method of screening a compound or its salt that alters the binding property between a ligand and the protein of the present invention, which comprises:

measuring the amount of a labeled ligand bound to the protein, when the labeled ligand is brought in contact with the protein of the present invention and when the labeled ligand and a test compound are brought in contact with the protein of the present invention, and,

comparing the binding property between them;

② a method of screening a compound or its salt that alters the binding property between a ligand and the protein of the present invention, which comprises:

measuring the amount of a labeled ligand bound to cells or the membrane fraction of the cells, when the labeled ligand is brought in contact with the cells or cell membrane fraction comprising the protein of the present invention and when the labeled ligand and a test compound are brought in contact with the cells or cell membrane fraction comprising the protein of the present invention, and,

comparing the binding property between them;

③ a method of screening a compound or its salt that alters the binding property between a ligand and the protein of the present invention, which comprises:

measuring the amount of a labeled ligand to the protein, when the labeled ligand is brought in contact with the protein expressed on the cell membrane induced by culturing a transformant comprising the DNA of the present invention and when the labeled ligand and a test compound are brought in contact with the protein of the present invention expressed on the cell membrane induced by culturing a transformant comprising the DNA of the present invention, and,

comparing the binding property between them;

④ a method of screening a compound or its salt that alters the binding property between a ligand and the protein of the present invention, which comprises:

measuring the receptor-mediated cell-stimulating activity (e.g., the activity that promotes or suppresses arachidonic acid release, acetylcholine release, intracellular Ca^{2+} release, intracellular cAMP production, intracellular cGMP production, inositol phosphate production, changes in cell membrane potential, phosphorylation of intracellular proteins, activation of c-fos, pH reduction, etc.), when a compound (e.g., a ligand to the protein of the present invention) that activates the protein of the present invention is brought in contact with cells comprising the protein of the present invention and when the compound that activates the protein of the present invention and a test compound are brought in contact with cells comprising the protein of the present invention, and,

comparing the binding property between them; and,

⑤ a method of screening a compound or its salt that alters the binding property between a ligand and the protein of the present invention, which comprises:

measuring the receptor-mediated cell-stimulating activity (e.g., the activity that promotes or suppresses arachidonic acid release, acetylcholine release, intracellular Ca^{2+} release, intracellular cAMP production, intracellular cGMP production, inositol phosphate production, changes in cell membrane potential, phosphorylation of intracellular proteins, activation of c-fos, pH reduction,

etc.), when a compound (e.g., a ligand for the protein of the present invention) that activates the protein of the present invention is brought in contact with the protein of the present invention expressed on the cell membrane induced by culturing a transformant comprising the DNA of the present invention and when the compound that activates the protein of the present invention and a test compound are brought in contact with the protein of the present invention expressed on the cell membrane induced by culturing a transformant comprising the DNA of the present invention, and,

comparing the binding property between them.

Before the protein of the present invention was obtained, it was required for screening G protein-coupled receptor agonists or antagonists to obtain candidate compounds first, using cells or tissues comprising the G protein-coupled receptor protein or the cell membrane fraction from rats or other animals (primary screening), and then examine the candidate compounds whether the compounds actually inhibit the binding between human G protein-coupled receptor protein and ligands (secondary screening). When cells, tissues, or the cell membrane fractions were directly used, it was practically difficult to screen agonists or antagonists to the objective protein, since other receptor proteins were present together.

However, using, for example, the human-derived protein of the present invention, the primary screening becomes unnecessary, and compounds that inhibit the binding between ligands and the G protein-coupled receptor protein can be efficiently screened. Furthermore, it is easy to assess whether the obtained compound is an agonist or antagonist.

Hereinafter, the screening methods of the present invention are described more specifically.

First, for the protein of the present invention used for the screening methods of the present invention, any substance may be used so long as it comprises the protein of the present invention described above. The cell membrane fraction from mammalian organs comprising the protein of the

present invention is preferred. However, it is very difficult to obtain human organs. It is thus preferable to use rat-derived receptor proteins or the like, produced by large-scale expression using recombinants.

To manufacture the protein of the present invention, the methods described above are used, and it is preferred to express the DNA of the present invention in mammalian and insect cells. For the DNA fragment encoding the objective protein region, the complementary DNA, but not necessarily limited thereto, is employed. For example, the gene fragments and synthetic DNA may also be used. To introduce a DNA fragment encoding the protein of the present invention into host animal cells and efficiently express the DNA there, it is preferred to insert the DNA fragment downstream of a polyhedorin promoter of nuclear polyhedrosis virus (NPV) belonging to baculovirus hosted by insects, SV40-derived promoter, retrovirus promoter, metallothionein promoter, human heat shock promoter, cytomegalovirus promoter, or SRα promoter. The amount and quality of the expressed receptor are examined by publicly known methods, for example, the method described in the literature [Nambi, P. et al., The Journal of Biological Chemistry (J. Biol. Chem.), 267, 19555-19559, 1992].

Therefore, in the screening methods of the present invention, the material that comprises the protein of the present invention may be the protein purified by publicly known methods, cells comprising the protein, or the cell membrane fraction comprising the protein.

In the screening methods of the present invention, when cells comprising the protein of the present invention are used, the cells may be fixed with glutaraldehyde, formalin, etc. The cells can be fixed by publicly known methods.

The cells comprising the protein of the present invention are host cells that express the protein. For the host cells, Escherichia coli, Bacillus subtilis, yeast, insect cells, animal cells and the like are preferred.

The cell membrane fraction refers to a fraction abundant in cell membrane obtained by cell disruption and subsequent

fractionation by a publicly known method. Useful cell disruption methods include cell squashing using a Potter-Elvehjem homogenizer, disruption using a Waring blender or Polytron (manufactured by Kinematica Inc.), disruption by ultrasonication, and disruption by cell spraying through thin nozzles under an increased pressure using a French press or the like. Cell membrane fractionation is effected mainly by fractionation using a centrifugal force, such as centrifugation for fractionation and density gradient centrifugation. For example, cell disruption fluid is centrifuged at a low speed (500 rpm to 3,000 rpm) for a short period of time (normally about 1 to about 10 minutes), the resulting supernatant is then centrifuged at a higher speed (15,000 rpm to 30,000 rpm) normally for 30 minutes to 2 hours. The precipitate thus obtained is used as the membrane fraction. The membrane fraction is rich in the protein expressed and membrane components such as cell-derived phospholipids and membrane proteins.

The amount of the protein in the cells comprising the protein and in the membrane fraction is preferably 10^3 to 10^8 molecules per cell, more preferably 10^5 to 10^7 molecules per cell. As the amount of expression increases, the ligand binding activity per unit of membrane fraction (specific activity) increases so that not only the highly sensitive screening system can be constructed but also large quantities of samples can be assayed with the same lot.

To screen the compounds that alter the binding property between ligands and the protein of the present invention described in ① to ③, for example, an appropriate protein fraction and a labeled ligand are necessary.

To screen the compounds that alter the binding property between ligands and the protein of the present invention described in ① to ③, for example, an appropriate protein fraction and a labeled ligand are necessary.

The protein fraction is preferably a fraction of naturally occurring receptor protein or a recombinant receptor protein fraction having an activity equivalent to that of the natural protein. Herein, the equivalent activity

is intended to mean a ligand binding activity, a signal transduction activity or the like that is equivalent to that possessed by naturally occurring proteins.

For the labeled ligand, a labeled ligand and a labeled ligand analogue are used. For example, ligands labeled with [^{3}H], [^{125}I], [^{14}C], [^{35}S], etc. are used.

Specifically, to screen the compounds that alter the binding property between ligands and the protein of the present invention, first, the protein standard is prepared by suspending cells or cell membrane fraction comprising the protein of the present invention in a buffer appropriate for the screening. For the buffer, any buffer that does not interfere with the binding of ligands to the protein is usable and examples of such a buffer are phosphate buffer, Tris-hydrochloride buffer, etc., having pH of 4 to 10 (preferably pH of 6 to 8). To minimize a non-specific binding, a surfactant such as CHAPS, Tween-80™ (Kao-Atlas Co.), digitonin, deoxycholate, etc. may be added to the buffer. To inhibit degradation of the receptor and ligands by proteases, protease inhibitors such as PMSF, leupeptin, E-64 (manufactured by Peptide Research Laboratory, Co.), and pepstatin may be added. To 0.01 to 10 ml of the receptor solution, a given amount (5,000 to 500,000 cpm) of labeled ligand is added, and 10^{-4} M - 10^{-10} M of a test compound is simultaneously added to be co-present. To examine non-specific binding (NSB), a reaction tube comprising an unlabeled test compound in a large excess is also prepared. The reaction is carried out at approximately 0 to 50°C, preferably about 4 to 37°C for about 20 minutes to about 24 hours, preferably about 30 minutes to about 3 hours. After completion of the reaction, the reaction mixture is filtrated through glass fiber filter paper, etc. and washed with an appropriate volume of the same buffer. The residual radioactivity on the glass fiber filter paper is then measured by means of a liquid scintillation counter or γ-counter. Regarding the count obtained by subtracting the amount of non-specific binding (NSB) from the count obtained in the absence of any competitive substance (B_0) as 100%,

when the amount of specific binding (B-NSB) is, for example, 50% or less, the test compound can be selected as a candidate substance having a potential of competitive inhibition.

To perform the methods ④ and ⑤ supra of screening the compounds that alter the binding property between ligands and the protein of the present invention, the protein-mediated cell-stimulating activity (e.g., activity that promotes or inhibits arachidonic acid release, acetylcholine release, intracellular Ca^{2+} release, intracellular cAMP production, intracellular cGMP production, inositol phosphate production, changes in cell membrane potential, phosphorylation of intracellular proteins, activation of c-fos, pH reduction, etc.) can be measured using publicly known methods or commercially available kits.

Specifically, the cells comprising the protein of the present invention are first cultured on a multi-well plate, etc. Prior to screening, the medium is replaced with fresh medium or with an appropriate non-cytotoxic buffer, followed by incubation for a given period of time in the presence of a test compound, etc. Subsequently, the cells are extracted or the supernatant is recovered and the resulting product is quantified by appropriate procedures. Where it is difficult to detect the production of the index substance (e.g., arachidonic acid) for the cell-stimulating activity due to a degrading enzyme comprised in the cells, an inhibitor against such a degrading enzyme may be added prior to the assay. For detecting activities such as the cAMP production suppression activity, the baseline production in the cells is increased by forskolin or the like and the suppressing effect on the increased baseline production may then be detected.

Screening by assaying the cell-stimulating activity requires cells that have expressed an appropriate protein. For the cells that have expressed the protein of the present invention, the cell line possessing the native protein of the present invention, the cell line expressing the

recombinant protein described above and the like are desirable.

For the test compound, for example, peptides, proteins, non-peptide compounds, synthetic compounds, fermentation products, cell extracts, plant extracts, and animal tissue extracts are used. These compounds may be novel or known compounds.

The kits for screening the compounds or their salts that alter the binding property between ligands and the protein of the present invention comprise the protein of the present invention, cells comprising the protein of the present invention, or the membrane fraction of cells comprising the protein of the present invention.

Examples of the screening kits of the present invention are as follow.

1. Reagents for screening

① Buffer for measurement and washing

Hanks' balanced salt solution (manufactured by Gibco Co.) supplemented with 0.05% bovine serum albumin (manufactured by Sigma Co.).

The solution is sterilized by filtration through a 0.45 μm filter, and stored at 4°C or may be prepared at use.

② Standard G protein-coupled receptor

CHO cells expressing the protein of the present invention are passaged in a 12-well plate at a density of 5×10^5 cells/well followed by culturing at 37°C under 5% CO_2 and 95% air for 2 days.

③ Labeled ligands

Aqueous solutions of ligands labeled with commercially available [^{3}H], [^{125}I], [^{14}C], [^{35}S], etc. are stored at 4°C or -20°C, and diluted to 1 μM with the measurement buffer.

④ Standard ligand solution

The ligand is dissolved in and adjusted to 1 mM with PBS comprising 0.1% bovine serum albumin (manufactured by Sigma Co.) and stored at -20°C.

2. Measurement method

① CHO cells expressing the protein of the present invention are cultured in a 12-well culture plate and washed

twice with 1 ml of the measurement buffer, and 490 µl of the measurement buffer is added to each well.

② After adding 5 µl of 10^{-3} - 10^{-10} M test compound solution, 5 µl of a labeled ligand is added to the mixture, and the cells are incubated at room temperature for an hour. To determine the amount of the non-specific binding, 5 µl of the non-labeled ligand is added in place of the test compound.

③ The reaction solution is removed, and the wells are washed 3 times with the washing buffer. The labeled ligand bound to the cells is dissolved in 0.2N NaOH-1% SDS, and mixed with 4 ml of liquid scintillator A (manufactured by Wako Pure Chemical Industries, Ltd.)

④ The radioactivity is measured using a liquid scintillation counter (manufactured by Beckman Co.), and the percent maximum binding (PMB) is calculated by the equation below.

$$PMB = [(B - NSB)/(B_0 - NSB)] \times 100$$

PMB : Percent maximum binding

B : Value obtained in the presence of a test compound

NSB : Non-specific binding

B_0 : Maximum binding

The compounds or their salts, which are obtainable using the screening methods or the screening kits of the present invention, are the compounds that alter the binding property between ligands and the protein of the present invention. Specifically, these compounds are: (a) compounds that have the G protein-coupled receptor-mediated cell-stimulating activity (e.g., activity that promotes or inhibits arachidonic acid release, acetylcholine release, intracellular Ca^{2+} release, intracellular cAMP production, intracellular cGMP production, inositol phosphate production, changes in cell membrane potential, phosphorylation of intracellular proteins, activation of c-fos, pH reduction, etc.) (so-called agonists to the protein of the present invention); (b) compounds having no cell stimulating-activity (so-called antagonists to the protein of the

present invention); (c) compounds that increase the binding affinity between ligands and the G protein-coupled protein of the present invention; and (d) compounds that reduce the binding affinity between ligands and the G protein-coupled protein of the present invention.

The compounds may be peptides, proteins, non-peptide compounds, synthetic compounds, fermentation products, and may be novel or known compounds.

Since agonists to the protein of the present invention have the same physiological activities as those of the ligands for the protein of the present invention, the agonists are useful as safe and low-toxic pharmaceuticals, correspondingly to the ligand activities (prophylactic and/or therapeutic agents for, e.g., central dysfunction (e.g., Alzheimer's disease, senile dementia, suppression of eating (anorexia), epilepsy, etc.), hormone diseases (e.g., weak pains, atonic bleeding, before and after expulsion, subinvolution of uterus, cesarean section, induced abortion, galactostasis, etc.), liver/gallbladder/pancreas/endocrine-associated diseases (e.g., diabetes mellitus, suppression of eating, etc.), inflammatory diseases (e.g., allergy, asthma, rheumatoid, etc.), circulatory diseases (e.g., hypertension, cardiac hypertrophy, angina pectoris, arteriosclerosis, etc.), respiratory system diseases (e.g., pneumonia, asthma, bronchitis, respiratory system infectious diseases, chronical and ooclusive lung disease, etc.), and infectious diseases (e.g., ichorrhemia, MRSA, respiratory system infectious disease, urinary tract infectious disease, biliary infectious disease, infetciositive enrteritis, tympanitis, prostatitis, etc.).

Further, since agonists to the protein of the present invention have activities similar to the physiological activities of ligands to the protein of the present invention, the agonists are particularly useful as safe and low-toxic prophylactic and/or therapeutic agents for treatment of digestive system diseases (e.g., enrteritis, diarrhea, coprostasis, malabsorption syndrome, etc.) depending upon the ligand activities.

Since antagonists to the protein of the present invention can suppress the physiological activities of

ligands to the protein of the present invention, the antagonists are useful as safe and low-toxic pharmaceuticals that inhibits the ligand activities (e.g., accommondational agents for hormonal secretion, central dysfunction caused of overproducing of ligand to the protein of the present invention, hormone diseases, liver/gallbladder/pancreas/endocrine-associated diseases (e.g., diabetes mellitus, suppression of eating, etc.), inflammatory diseases, circulatory diseases).

Since the antagonists to the protein of the present invention can suppress the physiological activities of ligands to the protein of the present invention, they are particularly useful as safe and low-toxic prophylactic and/or therapeutic agents, which inhibit the ligand activities, for treatment of digestive system diseases (e.g., enrteritis, diarrhea, coprostasis, malabsorption syndrome, etc.).

The compounds that reduce the binding affinity between ligands and the protein of the present invention are useful as safe and low-toxic pharmaceuticals that decrease the physiological activities of ligands to the protein of the present invention (prophylactic and/or therapeutic agents for, e.g., accommondational agents for hormonal secretion, central dysfunction caused of overproducing of ligand to the protein of the present invention, hormone diseases, liver/gallbladder/pancreas/endocrine-associated diseases (e.g., diabetes mellitus, suppression of eating, etc.), inflammatory diseases, circulatory diseases, and respiratory system diseases, infectious diseases, etc.).

Since the compounds that reduce the binding affinity between ligands and the protein of the present invention can suppress the physiological activities of the ligands to the protein of the present invention, they are particularly useful as prophylactic and/or therapeutic agents for treatment of digestive system diseases (e.g., enrteritis, diarrhea, coprostasis, malabsorption syndrome, etc.).

When compounds or their salt forms, which are obtainable by the screening methods or using the screening kits of the

present invention, are employed as ingredients of the pharmaceuticals described above, the compounds can be formulated in the pharmaceuticals in a conventional manner. For example, the compounds can be prepared into tablets, capsules, elixir, microcapsules, aseptic solution, suspension, etc., as described for pharmaceuticals comprising the protein of the present invention.

The preparations thus obtained are safe and low-toxic, and can be administered to, for example, human and mammals (e.g., rats, rabbits, sheep, swine, bovine, cats, dogs, monkeys, etc.).

The dose of the compounds or their salt forms varies depending on subject to be administered, target organs, conditions, routes for administration, etc.; in oral administration, e.g., for the adult patient, the dose is normally about 0.1 mg to about 100 mg, preferably about 1.0 to about 50 mg, and more preferably about 1.0 to about 20 mg per day (as 60 kg body weight). In parenteral administration, the single dose varies depending on subject to be administered, target organ, conditions, routes for administration, etc. but it is advantageous, e.g., for the adult patient suffering from a digestive disease, to administer the active ingredient intravenously in a daily dose of about 0.01 to about 30 mg, preferably about 0.1 to about 20 mg, and more preferably about 0.1 to about 10 mg (as 60 kg body weight). For other animal species, the corresponding dose as converted per 60 kg body weight can be administered.

(6) Quantification of the protein of the present invention, its partial peptide, or its salt form

The antibodies of the present invention are capable of specifically recognizing the protein of the present invention. Therefore, the antibodies can be used to quantify the protein of the present invention in a test fluid, especially for quantification by the sandwich immunoassay. That is, the present invention provides, for example, the following quantification methods:

(i) a method of quantifying the protein of the present invention in a test fluid, which comprises competitively reacting the antibody of the present invention with the test fluid and a labeled form of the protein of the present invention, and measuring the ratio of the labeled protein bound to the antibody; and,

(ii) a method of quantifying the protein of the present invention in a test fluid, which comprises reacting the test fluid with the antibody of the present invention immobilized on a carrier and a labeled form of the antibody of the present invention simultaneously or sequentially, and measuring the activity of the label on the immobilized carrier.

In (ii) described above, it is preferred that one antibody recognizes the N-terminal region of the protein of the present invention, and another antibody reacts with the C-terminal region of the protein of the present invention.

Using monoclonal antibodies to the protein of the present invention (hereinafter sometimes referred to as the monoclonal antibodies of the present invention), the protein of the present invention can be assayed and also detected by tissue staining or the like. For this purpose, an antibody molecule itself may be used, or $F(ab')_2$, Fab' or Fab fractions of the antibody molecule may also be used. Assay methods using antibodies to the protein of the present invention are not particularly limited. Any assay method can be used, so long as the amount of antibody, antigen, or antibody-antigen complex corresponding to the amount of antigen (e.g., the amount of the protein) in the test fluid can be detected by chemical or physical means and the amount of the antigen can be calculated from a standard curve prepared from standard solutions comprising known amounts of the antigen. For example, nephrometry, competitive methods, immunometric method, and sandwich method are appropriately used, with the sandwich method described below being most preferable in terms of sensitivity and specificity.

As the labeling agent for the methods using labeled substances, there are employed, for example, radioisotopes,

enzymes, fluorescent substances, luminescent substances, etc. For the radioisotope, for example, [^{125}I], [^{131}I], [^{3}H] and [^{14}C] are used. As the enzyme described above, stable enzymes with high specific activity are preferred; for example, β-galactosidase, β-glucosidase, alkaline phosphatase, peroxidase, malate dehydrogenase and the like are used. Example of the fluorescent substance used are fluorescamine and fluorescein isothiocyanate are used. For the luminescent substance, for example, luminol, luminol derivatives, luciferin, and lucigenin are used. Furthermore, the biotin-avidin system may be used for binding antibody or antigen to the label.

For immobilization of antigen or antibody, physical adsorption may be used. Chemical binding methods conventionally used for insolubilization or immobilization of proteins or enzymes may also be used. For the carrier, for example, insoluble polysaccharides such as agarose, dextran, cellulose, etc.; synthetic resin such as polystyrene, polyacrylamide, silicon, etc., and glass or the like are used.

In the sandwich method, the immobilized monoclonal antibody of the present invention is reacted with a test fluid (primary reaction), then with the labeled monoclonal antibody of the present invention (secondary reaction), and the activity of the label on the immobilizing carrier is measured, whereby the amount of the protein of the present invention in the test fluid can be quantified. The order of the primary and secondary reactions may be reversed, and the reactions may be performed simultaneously or with an interval. The methods of labeling and immobilization can be performed by the methods described above.

In the immunoassay by the sandwich method, the antibody used for immobilized or labeled antibodies is not necessarily one species, but a mixture of two or more species of antibody may be used to increase the measurement sensitivity.

In the methods of assaying the protein of the present invention by the sandwich method, antibodies that bind to

different sites of the protein are preferably used as the monoclonal antibodies of the present invention for the primary and secondary reactions. That is, in the antibodies used for the primary and secondary reactions are, for example, when the antibody used in the secondary reaction recognizes the C-terminal region of the protein, it is preferable to use the antibody recognizing the region other than the C-terminal region for the primary reaction, e.g., the antibody recognizing the N-terminal region.

The monoclonal antibodies of the present invention can be used for the assay systems other than the sandwich method, for example, competitive method, immunometric method, nephrometry, etc. In the competitive method, antigen in a test fluid and the labeled antigen are competitively reacted with antibody, and the unreacted labeled antigen (F) and the labeled antigen bound to the antibody (B) are separated (B/F separation). The amount of the label in B or F is measured, and the amount of the antigen in the test fluid is quantified. This reaction method includes a liquid phase method using a soluble antibody as an antibody, polyethylene glycol for B/F separation and a secondary antibody to the soluble antibody, and an immobilized method either using an immobilized antibody as the primary antibody, or using a soluble antibody as the primary antibody and immobilized antibody as the secondary antibody.

In the immunometric method, antigen in a test fluid and immobilized antigen are competitively reacted with a definite amount of labeled antibody, the immobilized phase is separated from the liquid phase, or antigen in a test fluid and an excess amount of labeled antibody are reacted, immobilized antigen is then added to bind the unreacted labeled antibody to the immobilized phase, and the immobilized phase is separated from the liquid phase. Then, the amount of the label in either phase is measured to quantify the antigen in the test fluid.

In the nephrometry, insoluble precipitate produced after the antigen-antibody reaction in gel or solution is quantified. When the amount of antigen in the test fluid is

small and only a small amount of precipitate is obtained, laser nephrometry using scattering of laser is advantageously employed.

For applying these immunological methods to the measurement methods of the present invention, any particular conditions or procedures are not required. Systems for measuring the protein of the present invention or its salts are constructed by adding the usual technical consideration in the art to the conventional conditions and procedures. For the details of these general technical means, reference can be made to the following reviews and texts. [For example, Hiroshi Irie, ed. "Radioimmunoassay" (Kodansha, published in 1974), Hiroshi Irie, ed. "Sequel to the Radioimmunoassay" (Kodansha, published in 1979), Eiji Ishikawa, et al. ed. "Enzyme immonoassay" (Igakushoin, published in 1978), Eiji Ishikawa, et al. ed. "Immunoenzyme assay" (2nd ed.) (Igakushoin, published in 1982), Eiji Ishikawa, et al. ed. "Immunoenzyme assay" (3rd ed.) (Igakushoin, published in 1987), Methods in ENZYMOLOGY, Vol. 70 (Immunochemical Techniques (Part A)), ibid., Vol. 73 (Immunochemical Techniques (Part B)), ibid., Vol. 74 (Immunochemical Techniques (Part C)), ibid., Vol. 84 (Immunochemical Techniques (Part D: Selected Immunoassays)), ibid., Vol. 92 (Immunochemical Techniques (Part E: Monoclonal Antibodies and General Immunoassay Methods)), ibid., Vol. 121 (Immunochemical Techniques (Part I: Hybridoma Technology and Monoclonal Antibodies))(all published by Academic Press Publishing).

As described above, the protein of the present invention or its salts can be quantified with high sensitivity, using the antibodies of the present invention.

By quantifying the protein of the present invention or its salts using the antibodies of the present invention, diagnosis can be made on various diseases.

The antibodies of the present invention can also be used for specifically detecting the protein of the present invention present in test samples such as body fluids or tissues. The antibodies may also be used for preparation of

antibody columns for purification of the present invention, for detection of the protein of the present invention in each fraction upon purification, and for analysis of the behavior of the protein of the present invention in the test cells.

(7) Preparation of non-human animals carrying the DNA encoding the G protein-coupled receptor protein of the present invention

Using the DNA of the present invention, non-human transgenic animals expressing the protein of the present invention can be prepared. Examples of the non-human animals include mammals (e.g., rats, mice, rabbits, sheep, swine, bovine, cats, dogs, monkeys, etc.) (hereinafter merely referred to as animals) can be used, with mice and rabbits being particularly appropriate.

To transfer the DNA of the present invention to target animals, it is generally advantageous to use the DNA in a gene construct ligated downstream of a promoter that can express the DNA in animal cells. For example, when the DNA of the present invention derived from rabbit is transferred, e.g., the gene construct, in which the DNA is ligated downstream of a promoter that can expresses the DNA of the present invention derived from animals comprising the DNA of the present invention highly homologous to the rabbit-derived DNA, is microinjected to rabbit fertilized ova; thus, the DNA-transferred animal, which is capable of producing a high level of the protein of the present invention, can be produced. Examples of the promoters that are usable include virus-derived promoters and ubiquitous expression promoters such as a metallothionein promoter, but promoters of NGF gene and enolase that are specifically expressed in the brain are preferably used.

The transfer of the DNA of the present invention at the fertilized egg cell stage secures the presence of the DNA in all germ and somatic cells in the produced animal. The presence of the protein of the present invention in the germ cells in the DNA-transferred animal means that all germ and

somatic cells comprise the protein of the present invention in all progenies of the animal. The progenies of the animal that took over the gene comprise the protein of the present invention in all germ and somatic cells.

The DNA-transferred animals of the present invention can be maintained and bled in the conventional environment as animals carrying the DNA after confirming the stable retention of the gene in the animals through mating. Furthermore, mating male and female animals comprising the objective DNA results in acquiring homozygote animals having the transferred gene on both homologous chromosomes. By mating the male and female homozygotes, bleeding can be performed so that all progenies comprise the DNA.

Since the protein of the present invention is highly expressed in the animals in which the DNA of the present invention has been transferred, the animals are useful for screening of agonists or antagonists to the protein of the present invention.

The animals in which the DNA of the present invention has been transferred can also be used as cell sources for tissue culture. The protein of the present invention can be analyzed by, for example, directly analyzing the DNA or RNA in tissues from the mouse in which the DNA of the present invention has been transferred, or by analyzing tissues comprising the protein expressed from the gene. Cells from tissues comprising the protein of the present invention are cultured by the standard tissue culture technique. Using these cells, for example, the function of tissue cells such as cells derived from the brain or peripheral tissues, which are generally difficult to culture, can be studied. Using these cells, for example, it is possible to select pharmaceuticals that increase various tissue functions. When a highly expressing cell line is available, the protein of the present invention can be isolated and purified from the cell line.

In the specification and drawings, the codes of bases and amino acids are denoted in accordance with the IUPAC-IUB Commission on Biochemical Nomenclature or by the common

codes in the art, examples of which are shown below. For amino acids that may have the optical isomer, L form is presented unless otherwise indicated.

DNA : deoxyribonucleic acid
cDNA: complementary deoxyribonucleic acid
A : adenine
T : thymine
G : guanine
C : cytosine
RNA : ribonucleic acid
mRNA : messenger ribonucleic acid
dATP : deoxyadenosine triphosphate
dTTP : deoxythymidine triphosphate
dGTP : deoxyguanosine triphosphate
dCTP : deoxycytidine triphosphate
Gly or G : glycine
Ala or A: alanine
Val or V: valine
Leu or L : leucine
Ile or I: isoleucine
Ser or S : serine
Thr or T : threonine
Cys or C : cysteine
Met or M : methionine
Glu or E : glutamic acid
Asp or D : aspartic acid
Lys or K : lysine
Arg or R: arginine
His or H: histidine
Phe or F : phenylalanine
Tyr or Y: tyrosine
Trp or W : tryptophan
Pro or P : proline
Asn or N : asparagine
Gln or Q : glutamine
pGlu : pyroglutamic acid
Xaa : undefine amino acid residue
Tos : p-toluenesulfonyl

Bzl : benzyl
Cl_2Bzl: 2,6-dichlobenzyl
Bom: benzyloxymethyl
Z: benzyloxycarbonyl
Cl-Z : 2-chlorobenzyloxycarbonyl
Br-Z : 2-bromobenzyloxycarbonyl
Boc : t-butoxycarbonyl
DNP : dinitrophenol
Trt : trityl
Bum : t-butoxymethyl
Fmoc : N-9-fluorenylmethoxycarbonyl
HOBt : 1-hydroxybenztriazole
HOOBt: 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine
HONB : 1-hydroxy-5-norbornene-2,3-dicarboximide
DCC : N,N'-dicyclohexylcarbodiimide
ATP : Adenosine triphosphate
EDTA : ethylenediamine tetraacetic acid
SDS : sodium dodecyl sulfate

The sequence identification numbers in the sequence listing of the specification indicates the following sequence, respectively.

[SEQ ID NO:1]

This shows the amino acid sequence of the protein derived from human brain.

[SEQ ID NO:2]

This shows the base sequence of DNA encoding human brain-derived protein having the amino acid sequence shown by SEQ ID NO:1(ZAQC).

[SEQ ID NO:3]

This shows the base sequence of DNA encoding human brain- derived protein having the amino acid sequence shown by SEQ ID NO:1(ZAQT).

[SEQ ID NO:4]

This shows the base sequence of primer 1 used in Example 1.

[SEQ ID NO:5]

This shows the base sequence of primer 2 used in Example 1.

[SEQ ID NO:6]

This shows the base sequence of primer 3 used in Example 2.

[SEQ ID NO:7]

This shows the base sequence of primer 4 used in Example 2.

[SEQ ID NO:8]

This shows the base sequence of ZAQ probe used in Example 2.

[SEQ ID NO:9]

This shows the base sequence of ZAQC Sal primer used in Example 2.

[SEQ ID NO:10]

This shows the base sequence of ZAQC Spe primer used in Example 2.

[SEQ ID NO:11]

This shows the amino acid sequence of the N-terminus of the purified ZAQ activated peptide in Example 3 (3-8).

[SEQ ID NO:12]

This shows the base sequence of primer ZF1 in Example 4.

[SEQ ID NO:13]

This shows the base sequence of the primer ZF2 in Example 4.

[SEQ ID NO:14]

This shows the base sequence of the primer ZF3 in Example 4.

[SEQ ID NO:15]

This shows the 3'-terminus of base sequence of DNA encoding Human type ZAQ ligand peptide in Example 4.

[SEQ ID NO:16]

This shows the base sequence of primer ZAQL-CF in Example 4.

[SEQ ID NO:17]

This shows the base sequence of primer ZAQAL-XR1 in Example 4.

[SEQ ID NO:18]

This shows the base sequence of DNA fragment, which was obtained in Example 4.

[SEQ ID NO:19]

This shows the base sequence of DNA fragment, which was obtained in Example 4.

[SEQ ID NO:20]

This shows the amino acid sequence of Human type ZAQ ligand maturation peptide.

[SEQ ID NO:21]

This shows the amino acid sequence of Human type ZAQ ligand mature peptide.

[SEQ ID NO:22]

This shows the amino acid sequence of Human type ZAQ ligand precursor peptide.

[SEQ ID NO:23]

This shows the amino acid sequence of Human type ZAQ ligand precursor peptide.

[SEQ ID NO:24]

This shows the base sequence of DNA which has DNA encoding Human type ZAQ ligand precursor peptide represented by SEQ ID NO:28.

[SEQ ID NO:25]

This shows the base sequence of DNA which has DNA encoding Human type ZAQ ligand precursor peptide represented by SEQ ID NO:29.

[SEQ ID NO:26]

This shows the base sequence of DNA which has DNA encoding Human type ZAQ ligand mature peptide represented by SEQ ID NO:20.

[SEQ ID NO:27]

This shows the base sequence of DNA which has DNA encoding Human type ZAQ ligand mature peptide represented by SEQ ID NO:21.

[SEQ ID NO:28]

This shows the base sequence of DNA which has DNA encoding Human type ZAQ ligand precursor peptide represented by SEQ ID NO:22.

[SEQ ID NO:29]

This shows the base sequence of DNA which has DNA encoding Human type ZAQ ligand precursor peptide represented by SEQ ID NO:23.

[SEQ ID NO:30]

This shows the base sequence of DNA fragment, which was obtained in Example 5(5-1).

[SEQ ID NO:31]

This shows the amino acid sequence of the N-terminus of the Human type ZAQ ligand peptide in Example 6 (6-2).

Escherichia coli DH5α/pCR2.1-ZAQC obtained in Example 1 later described was on deposit with the Ministry of International Trade and Industry, Agency of Industrial Science and Technology, National Institute of Bioscience and Human Technology (NIBH), located at 1-1-3, Higashi, Tsukuba-shi, Ibaraki, Japan, as the Accession Number FERM BP-6855 on August 23, 1999 and with Institute for Fermentation, Osaka (IFO), located at 2-17-85, Juso-honmachi, Yodogawa-ku, Osaka-shi, Osaka, Japan, as the Accession Number IFO 16301 on August 4, 1999.

Escherichia coli DH5α/pCR2.1-ZAQT obtained in Example 1 later described was on deposit with the Ministry of International Trade and Industry, Agency of Industrial Science and Technology, National Institute of Bioscience and Human Technology (NIBH) as the Accession Number FERM BP-6856 on August 23, 1999 and with Institute for Fermentation, Osaka (IFO) as the Accession Number IFO 16302 on August 4, 1999.

Escherichia coli TOP10/pHMITA obtained in Example 4 later described was on deposit with the Ministry of International Trade and Industry, Agency of Industrial Science and Technology, National Institute of Bioscience and Human Technology (NIBH) as the Accession Number FERM BP-7219 on July 13, 2000 and with Institute for Fermentation, Osaka (IFO) as the Accession Number IFO 16440 on May 26, 2000.

Escherichia coli TOP10/pHMITG obtained in Example 4 later described was on deposit with the Ministry of International Trade and Industry, Agency of Industrial Science and

Technology, National Institute of Bioscience and Human Technology (NIBH) as the Accession Number FERM BP-7220 on July 13, 2000 and with Institute for Fermentation, Osaka (IFO) as the Accession Number IFO 16441 on May 26, 2000.

The present invention is described in detail below with reference to REFERENCE EXAMPLES and EXAMPLES, but is not deemed to limit the scope of the present invention thereto. The gene manipulation procedures using Escherichia coli were performed according to the methods described in the Molecular Cloning.

EXAMPLE 1: Cloning of the cDNA encoding the human brain-derived G protein-coupled receptor protein (ZAQ) and determination of the base sequence

Using human fetal brain cDNA (CLONTECH Inc.) as a template and two primers, namely, primer 1 (5'-GTC GAC ATG GAG ACC ACC ATG GGG TTC ATG G -3'; SEQ ID NO:4) and primer 2 (5'-ACT AGT TTA TTT TAG TCT GAT GCA GTC CAC CTC TTC -3'; SEQ ID NO:5), a PCR reaction was carried out. The reaction solution in the above reaction comprised 1/10 volume of the cDNA for the template, 1/50 volume of Advantage 2 Polymerase Mix (CLONTECH Inc.), 0.2 μM of primer 1, 0.2 μM of primer 2, 200 μM of dNTPs and a buffer attached to the enzyme to make the final volume 25 μl. In the PCR reaction, after (1) the reaction solution was heated at 94°C for 2 minute, (2) a cycle of heating at 94°C for 20 seconds followed by 72°C for 100 seconds, was repeated 3 times, (3) a cycle of heating at 94°C for 20 seconds followed by 68°C for 100 seconds, was repeated 3 times, (4) a cycle of heating at 94°C for 20 seconds followed by 64°C for 20 seconds and 68°C for 100 seconds, was repeated 38 times, and (5) finally, an extension reaction was carried out at 68°C for 7 minutes. After completion of the PCR reaction, the reaction product was subcloned to plasmid vector pCR2.1 (Invitrogen Inc.) according to the instructions attached to the TA cloning kit (Invitrogen Inc). Then, it was introduced into Escherichia coli DH5α, and the clones comprising the cDNA were selected on LB agar plates comprising ampicillin. The sequence of

each clone was analyzed to give two of the cDNA sequences encoding the novel G protein-coupled receptor protein, i.e., ZAQC (SEQ ID NO:2) and ZAQT (SEQ ID NO:3). The novel G protein-coupled receptor protein having the amino acid sequence deduced therefrom were designated ZAQ since they have the same base sequence (SEQ ID NO:1). The transformant which contains the DNA represented by SEQ ID NO:2, was designated Escherichia coli DH5α/pCR2.1-ZAQC. The transformant which contains the DNA represented by SEQ ID NO:3, was designated Escherichia coli DH5α/pCR2.1-ZAQT.

Example 2: Analysis of distribution of ZAQ expression by TaqMan PCR

As primers and a probe to be used in the TaqMan PCR, Primer 3 (5′- TCATGTTGCTCCACTGGAAGG -3′ SEQ ID (NO:6)), Primer 4 (5′- CCAATTGTCTTGAGGTCCAGG -3′ (SEQ ID NO:7)) and ZAQ probe (5′- TTCTTACAATGGCGGTAAGTCCAGTGCAG-3′ (SEQ ID NO:8)) were designed using Primer Express Ver.1.0 (PE Biosystems Japan). FAM (6-carboxyfluorescein) was added as a reporter dye.

Standard DNA was prepared by following: The PCR fragment was amplified using pAK-ZAQC as a template, and Primer ZAQC Sal (5′-GTCGACATGGAGACCACCATGGGGTTCATGG -3′ (SEQ ID NO:9))and Primer ZAQC Spe (5′-ACTAGTTTATTTTAGTCTGATGCAGTCCACCTCTTC-3′ (SEQ ID NO:10)), purified with CHROMA SPIN200 (CLONTECH Laboratories, Inc., CA,USA), and then adjusted to have a concentration of 10^0-10^6 copies/μl at use. Human Tissue cDNA Panel I and Panel II (CLONTECH Laboratories, Inc., Ca, USA) were used as a cDNA source of each tissue. To the primers, probe and template, TaqMan Universal PCR Master Mix (PE Biosystems Japan) was added in given amount which is described in the attached instructions, and then a PCR reaction and analysis were performed with ABI PRISM 7700 Sequence Detection System (PE Biosystems Japan).. The results are shown in Figure 8 and Table 1. The expression of ZAQC was found mainly in the testis, and then in the sites such as lung and brain.

Table 1

Tissue	ZAQ (copies/μl)
Brain	6.1
Heart	2.9
Kidney	2.8
Liver	2.6
Lung	7.0
Pancreas	2.1
Placenta	3.2
Skeletal muscle	2.6
Colon	1.8
Ovary	3.4
Leukocyte	0.0
Prostate	0.7
Small intestine	2.2
Spleen	2.1
Testis	28.0
Thymus	1.1

Example 3: Isolation of the ZAQ-activating peptide

(3-1) Preparation of the milk extract solution

Using milk pasteurized at a low temperature, which was commercially available, the following procedures were performed to prepare an extract solution. Two litters of milk were centrifuged at 10,000rpm for 15 minutes at 4°C with a high-speed centrifuge (CR26H R10A rotor: Hitachi System Engineering Co., Ltd.). The obtained supernatant was filtered through gauze to remove lipids. Acetic acid was added thereto to adjust the concentration to become 1M at final concentration, and the mixture was agitated for 30 minutes at 4°C. Then, the mixture was centrifuged at 10,000rpm for 15 minutes with a high-speed centrifuge (CR26H R10A rotor: Hitachi System Engineering Co., Ltd.). The obtained supernatant was filtered to remove contaminants. Acetone was added thereto as twice as much volume of the supernatant with agitation for 3 hours at 4°C. Then, it was

centrifuged at 10,000rpm for 15 minutes with a high-speed centrifuge (CR26H R10A rotor: Hitachi System Engineering Co., Ltd.). The obtained supernatant was filtered to remove contaminants. The obtained supernatant was evaporated with a rotary evaporator to remove acetone from the solution and concentrated into 1350 ml at final volume. 675 ml each of the obtained solution was mixed with 338 ml of diethyl ether with vigorous shaking in a separatory funnel. After, they were separated into two phases, the aqueous phase was collected. The same procedure was repeated once to obtain a clear aqueous solution. The aqueous solution obtained was concentrated upto 800 ml using a rotary evaporator, to obtain a final extract.

(3-2) Rough fractionation of the milk extract solution using C18 reversed phase chromatography

Methanol was added into a 10 g of Sep-Pak C18 (Waters) column filled with silica gel to which octadecyl group was fixed, to swell the gel. Then, it was equilibrated with 1 M acetic acid. The extract solution prepared in (3-1) (the extract solution from 2-litter of milk) was loaded onto the column. Then, 100 ml of acetic acid (1 M) was added into the column to wash the gel. Then, into the column, 200 ml of 60% acetonitrile/0.1% trifuluoroacetic acid was added to elute the desired crude peptide. The obtained solution was concentrated with a rotary evaporator, and then lyophilized with a lyophilizor (12E1; Virtis).

(3-3) Rough fractionation of the milk extract solution using Sulfopropyl ion exchange chromatography

SP Sephadex C-25 (Amersham Pharmacia Biotech) swollen with 100 mM HCl, was loaded onto the column made of polypropylene at a volume of 2 ml. The column was washed with distilled water and ammonium formate (pH 4.0), and equilibrated with Solution I (2 M anmmonium formate:acetonitrile:water = 1:25:74). The lyophilized product obtained in (3-2) was dissolved in 20 ml of Solution I, and loaded onto 2 ml SP Sephadex C-25. After the column

was washed with 10 ml of solution I, the peptide was eluted with 10 ml each of Solution II (2 M anmmonium formate:acetonitrile:water = 1:2.5:6.5), Solution III (2M ammonium formate:acetonitrile: water = 1:1:2) and Solution IV (2M ammonium formate:acetonitrile: water = 1:0.5:0.5) in this order. Each of the eluted solutions was lyophilized with a lyophilizer (12EL; VirTis).

(3-4) Fractionation of the milk extract using TSKgel ODS80Ts reversed phase high performance liquid chromatography

The column for TSKgel ODS80Ts reversed phase high performance liquid chromatography (Tosoh,4.6 mm x 25 cm) was equilibrated with 81.7 vol.% of Solution A (0.1% trifuluoroacetyl/distilled water) and 8.3 vol.% of Solution B(trifuluoroacetyl/60% acetonitrile) at a flow rate of 1 ml/min at 40°C. The lyophilized products (I - IV) obtained in (3-3) were dissolved in 4 ml of 1 M acetic acid, and then subjected to chromatography treatment. 4 ml of the solution of the lyophilized products was loaded onto the said column, and eluted with Solution A and Solution B at a flow rate of 1 ml/min while changing a Solution A/Solution B ratio to 67 vol.% Solution A/33 vol.% Solution B for 1 minute, followed by changing the ratio from 67 vol.% Solution A/33 vol.% Solution B to 0 vol.% Solution A/100 vol.% Solution B for the next 40 minutes in a linear gradient manner.

1ml each of the eluted solution was taken, and a fraction number was given to each fraction. Each fraction (2 μl) was mixed with 0.2% Bovine Serum Albumin (BSA)/distilled water (150 μl) and lyophilized. These lyophilized products were used as samples for the assay measuring the activity of increasing intracellular Ca-ion concentration described in (3-5) as described below.

(3-5) Measurement of activity of increasing intracellular Ca-ion concentration with FLIPR

ZAQ stable expression cell lines were prepared as follows: That is, one clone of DH5α/pCR2.1-ZAQC obtained in Example 1 was cultured in LB medium comprising ampicillin

with vigorous shaking to obtain a plasmid (pCR2.1-ZAQC). The plasmid was digested with restriction enzymes (Sal I and Spe I), in order to obtain an insert encoding ZAQC. Then, pAKKO-1.11H was also digested with Sal I and Spe I for ligation with said plasmid with ligation Express Kit (CLONTECH Laboratories, Inc (CA, USA)), and they were introduced into E.coli DH10B by an electroporation method. The structure of the plasmid contained in the obtained clone was analyzed using restriction enzyme treatment and sequence analysis. The one with correct structure was used as a CHO cell expression plasmid, pAK-ZAQC.

This plasmid (pAK-ZAQC) was transfected into CHO/dfhr$^-$ cell (American Type Culture Collection) using CellPhect Transfection Kit (Amersham Pharmacia Biotech). First, the plasmid DNA (4 μg) was dissolved in 120 μl of distilled water, and 120 µl of Buffer A (CellPhect Transfection Kit) was added thereto. The mixture was agitated, and allowed to stand for 10 minutes. 240 µl of Buffer B (CellPhect Transfection Kit) was added thereto, and the mixture was agitated vigorously to form DNA-Calcium phosphate complex comprising said DNA. 5 x 10^5 of CHO/dhfr$^-$ cells were inoculated on 60 mm Petri's dish, and cultured in Ham's F-12 medium (Nissui Pharmaceutical co.) comprising 10% of fetal bovine serum (BIO WHITTAKER) at 37°C under 5% CO_2 for one day. Then, 480 µl of the suspension of the DNA-Calcium phosphate complex was added dropwise to the cells on the Petri's dish. It was cultured at 37°C under 5% CO_2 for 6 hours. Then, the cells were washed twice with Ham's F-12 medium, which did not comprise the fetal bovine serum. 1.2 ml of buffer (140 mM NaCl, 25 mM HEPES, 1.4 mM Na_2HPO_4, pH7.1) comprising 15% of glycerol was added to the cells in the dish and treated for 2 minutes. The cells were washed twice with Ham's F-12 medium without the serum again. Then, it was cultured in Ham's F-12 medium comprising 10% of fetal bovine serum at 37°C under 5% CO_2 overnight. Said cells were treated with trypsin for dispersion, and recovered from the dish. The cells (2 x 10^4) were inoculated in a 6-well plate. Culturing was initiated in Dulbecco's modified Eagle

medium (DMEM) comprising 10% of dialyzed fetal bovine serum (JRH BIOSCIENCES), 1 mM MEM non-amino acid solution (Dainippon pharmaceutical), 100 units/ml Penicillin and 100 μg/ml Streptomycin at 37°C under 5% CO_2. The transformed CHO cells into which the plasmid was introduced, were able to survive in said medium, and non-tranfected cells died gradually. Thus, the medium was exchanged to remove the dead cells after the first day and the second day. About 21 colonies of the transformed CHO cells grown after the eighth to tenth day of culturing, were selected. RNA was recovered from each of selected cells using an RNA isolation kit, which is commercially available. Publicly known RT-PCR method was used to select ZAQ expression CHO cell B-1 clone (hereinafter, referred to as ZAQC-B1) which showed high expression of ZAQ.

As a control, ETA (endothelin A receptor)-expressing CHO cell No.24 clone (hereinafter referred to as ETA24 cell. Journal of Pharmacology and Experimental Therapeutics, 279; 675-685,1996) was used.

The activity of increasing intracellular Ca-ion concentration of ZAQC-B1 cells or ETA24 cells was assayed for the samples obtained in above (3-4) using the FLIPR (Molecular Devices). The ZAQC-B1 cells and ETA24 cells subcultured in DMEM supplemented with 10% dialyzed fetal bovine serum (hereinafter referred to as d FBS), were used. The ZAQC-B1 cells and ETA24 cells were suspended respectively in the medium (10% d FBS-DMEM) to adjust the concentration to 15 x 10^4 cells/ml. 200 μl of the cells were inoculated to each well (3.0 x 10^4 cells /200 μl/well) in FLIPR 96-well plate (Black plate clear bottom, Coster), and incubated in an incubator at 37°C under 5% CO_2 overnight, and then, the cells obtained (hereinafter referred as to cell plate) were used. 20 ml of H/HBS (9.8 g of Nissui Hanks 2, 0.35 g of sodium bicarbobonate, 4.77 g HEPES, adjusted to pH 7.4 with sodium hydroxide, sterilized with a sterilizing filter), 200 μl of 250 mM Probenecid, and 200 μl of fetal bovine serum (FBS) were mixed. 2 vials (50μg) of Fluo 3-AM (Dojin chemical research institute) was dissolved

in 40 µl of dimethyl sulfoxide and 40 µl of 20% Pluronic acid (Molecular Probes), and added to the above H/HBSS-Probenecid-FBS solution. After mixing these, the medium was removed from the cell plate, and 100 µl of the mixture was poured into each well of the cell plate using an 8-well pipette. Then, the cell plate was incubated at 37°C under 5% CO_2 for an hour (dye loading). For the samples for assay which was obtained in Example (3-4), 150 µl H/HBSS comprising 2.5mM Probenecid and 0.2% BSA was added to each fraction to dilute. They were transferred to FLIPR 96-well plate (V-Bottom plate, Coster, hereinafter referred as to sample plate). After completion of dye loading, the cell plate was washed 4 times with the washing buffer (H/HBSS to which 2.5 mM Probenedid was added) by using a plate washer (Molecular Devices). 100 µl of the washing buffer was saved for further procedures. This cell plate and sample plate were loaded onto FLIPR to conduct an assay. (By FLIPR, 50 µl of sample was transferred from the sample plate to the cell plate.)

As a result, the activity of increasing intracellular Ca-ion concentration specific to ZAQC-B1 cells was found in Fraction No.53 that was obtained by conducting a reversed phase high performance liquid chromatography in (3-4) for Solution IV (3-3).

(3-6) Purification using TSKgel Super-Phenyl reversed phase high performance liquid chromatography

(1) The column for TSKgel Super-Phenyl reversed phase high performance liquid chromatography (Tosoh,0.46 cm x 10 cm) was equilibrated with 81.7 vol.% of Solution A (0.1% trifuluoroacetic acid/distilled water)/8.3 vol.% of Solution B (0.1% trifuluoroacetic acid/60% acetonitrile) at a flow rate of 1 ml/min at 40°C. The chromatography was conducted for Fraction No.53 obtained in (3-4). That is, 1 ml of Fraction No.53 was loaded onto said column, and eluted with Solution A and Solution B at a flow rate of 1 ml/min while changing a Solution A/Solution B ratio to 75 vol.% Solution A/25 vol.% Solution B for 1 minute, followed by changing the

ratio to 67 vol.% Solution A/33 vol.% Solution B for the next 75 minutes in a linear gradient manner.

500 µl each of the eluted solution was collected, and a fraction number was given to each fraction. 25 µl of each fraction was mixed with 150 µl of 0.2 % BSA, and lyophilized with a lyophilizer (12EL;Vir Tis). 150 µl of H/HBSS comprising 2.5 mM Probenecid was added to the lyophilized product to dissolve it. To measure the function of receptor activation with respect to ZAQC-B1 cells, the activity of increasing intracellular Ca ion concentration was measured by the method according to (3-5) by using 50 µl of this solution. As a result, the components having the function of receptor activation with respect to the target ZAQC cells, i.e., ZAQ activating components, were eluted mainly in Fraction Nos. 103-105.

(3-7) Purification using µRPC C2/C18 ST4.6/100 reversed phase high performance liquid chromatography

The column for µRPC C2/C18 ST4.6/100 reversed high performance liquid chromatography (Amersham Pharmacia Biotech, 0.46 cm x 10 cm) was equilibrated with 95 vol.% Solution A (0.1% heptafluorobytyric acid/distilled water)/5 vol.% solution B (0.1% heptafluorobutyric acid/100% acetonitrile) at a flow rate of 1 ml/min at 40°C.

After Fractions Nos. 103-105 selected from the divided fractions obtained in TSKgel Super-Phenyl reversed phase high performance liquid chromatography were loaded onto µRPC C2/C18 ST4.6/100 reversed phase column, and eluted with Solution A (0.1% heptafluorobutyric acid/distilled water) and Solution B (0.1% heptafluorobutyric acid/100% acetonitrile) at a flow rate of 1 ml/min while quickly changing a Solution A/Solution B ratio from 95 vol.% Solution A/5 vol.% Solution B to 65 vol.% Solution A/35 vol.% Solution B for 1 minute, followed by changing the ratio to 50 vol.% Solution A/50 vol.% Solution B for the next 60 minutes in a linear gradient manner, to recover the eluted solution. The eluted solution was detected as a single peak at 210 nm ultraviolet absorption.

500 µl each of the eluted solution was collected and a fraction number was given to each fraction. 10 µl each of the fractions was mixed with 150 µl of 0.2 % BSA, and lyophilized with a lyophilizer (12EL;Vir Tis). 150 µl of H/HBSS comprising 2.5mM Probenecid was added to the lyophilized products to dissolve them. To measure the function of receptor activation with respect to ZAQC-B1 cells, 50 µl of this solution was used according to the above testing method (3-5). As a result, the components having the function of receptor activation with respect to the target ZAQC cells, i.e., ZAQ activating components, were eluted mainly in Fractions Nos. 82-84. This activation peak was matched completely to the ultraviolet absorption peak at 210 nm, leading to the conclusion that the product was purified enough to obtain the single peptide.

(3-8) Analysis of the structure of purified ZAQ activating peptide

The following method was used to determine the structure of ZAQ-activating components obtained in Example (3-7). The solvent in the sample of the purified ZAQ activating components was removed with a Savant speed vac concentrator, and then, the dry solid product obtained was dissolved in DMSO (dimethyl sulfoxide). A portion of this solution was analyzed by the analysis of amino acid sequence to sequence from N-terminus using a protein sequencer (Perkin Elmer, PE Biosystems Procise 491cLC). As a result, out of the amino acid residues from N-terminus to the 16th amino acid residue, 14 residues could be identified (Ala Val Ile Thr Gly Ala Xaa Glu Arg Asp Val Gln Xaa Arg Ala Gly (SEQ ID NO:11; Xaa is a non-identified residue.).

Example 4: cDNA cloning for Human type ZAQ ligand peptide

A Blast search was conducted using, as a query, the N-terminus of amino acid sequence of the purified ZAQ activating peptide extracted from milk in Example 3. As a result, Human EST (X40467) was discovered, which has the same base sequence of DNA encoding the peptide having the

amino acid sequence represented by SEQ ID NO:11. This base sequence did not have a complete open reading frame. Thus, non-identified sequence was identified by RACE method to obtain the complete open reading frame. Then, the cDNA clone having the complete open reading frame was obtained.

From the information of EST (X40467), Primer ZF1 (SEQ ID NO:12), ZF2 (SEQ ID NO:13) and ZF3 (SEQ ID NO:14) were designed, and 3'RACE was conducted using human testis Marathon-ready cDNA (CLONTECH) as a template.

ZF1: 5'-GGTGCCACGCGAGTCTCAATCATGCTCC-3' (SEQ ID NO:12)
ZF2: 5'-GGGGCCTGTGAGCGGGATGTCCAGTGTG-3' (SEQ ID NO:13)
ZF3: 5'-CTTCTTCAGGAAACGCAAGCACCACACC-3' (SEQ ID NO:14)

A PCR reaction solution for 3'RACE was prepared by mixing 1 µl of 50 x Advandage 2 Polymerase Mix (CLONTECH), 5 µl of 10 x Advantage 2 PCR buffer attached (400 mM Tricine-KOH, 150 mM KOAc, 35 mM $Mg(OAc)_2$, 37.5 µg/ml BSA, 0.05% Tween-20, 0.05% Nonidet-P40), 4 µl of dNTP mixture (2.5mM each, TaKaRa Shuzo), 1 µl of 10 µM primer ZF1, 1 µl of 10µM primer AP1 (Primer AP1 was attached to Human testis Marathon-Ready cDNA Kit by CLONTECH), 5 µl of template cDNA (CLONTECH, Human testis Marathon-Ready cDNA)and 33 µl distilled water. The reaction was carried out under the conditions: (1) heating at 94°C for 60 seconds for denature, (2) repeating 5 times a cycle of heating at 94°C for 30 seconds followed by 72°C for 4 minutes, (3) repeating 5 times a cycle of heating at 94°C for 30 seconds followed by 70°C for 4 minutes, and (4) repeating 25 times a cycle of heating at 94°C for 30 seconds followed by 68°C for 44 minutes.

Then, Nested PCR was conducted using the reaction mixture of said PCR reaction as a template. The reaction solution was prepared by mixing 1 µl of 50 x Advandage 2 Polymerase Mix (CLONTECH), 5 µl of 10x Advantage 2 PCR buffer attached (400 mM Tricine-KOH, 150 mM KOAc,35 mM $Mg(OAc)_2$, 37.5 µg/ml BSA, 0.05% Tween-20, 0.05% Nonidet-P40), 4 µl of dNTP mixture (2.5 mM each, TaKaRa Shuzo), 1 µl of 10 µM primer ZF2, 1 µl of 10 µM primer AP2 (Primer AP2 was attached to Human testis Marathon-Ready cDNA Kit by

CLONTECH), 5 µl of template DNA (x50 said PCR reaction mixture)and 33 µl distilled water. The reaction was carried out under the conditions: (1) denaturing at 94°C for 60 seconds, (2) repeating 5 times a cycle of heating at 94°C for 30 seconds followed by 72°C for 4 minutes, (3) repeating 5 times a cycle of heating at 94°C for 30 seconds followed by 70°C for 4 minutes, and (4) repeating 25 times a cycle of heating at 94°C for 30 seconds followed by 68°C for 44 minutes.

Furthermore, the second nested PCR was conducted using the reaction solution of said PCR reaction as a template. The reaction solution was prepared by mixing 1 µl of 50 x Advandage 2 Polymerase Mix (CLONTECH), 5 µl of 10 x Advantage 2 PCR buffer attached (400 mM Tricine-KOH, 150 mM KOAc,35 mM $Mg(OAc)_2$, 37.5 µg/ml BSA, 0.05% Tween-20, 0.05% Nonidet-P40), 4 µl of dNTP mixture (2.5 mM each, TaKaRa Shuzρ), 1 µl of 10 µM primer ZF3, 1 µl of 10 µM primer AP2 (Primer AP2 was attached to Human testis Marathon-Ready DNA Kit by CLONTECH), 5 µl of template cDNA (x50 said PCR reaction mixture)and 33 µl distilled water. The reaction was carried out under the conditions: (1) denaturing at 94°C for 60 seconds, (2) repeating 5 times a cycle of heating at 94°C for 30 seconds followed by 72°C for 4 minutes, (3) repeating 5 times a cycle of heating at 94°C for 30 seconds followed by 70°C for 4 minutes, and (4) repeating 25 times a cycle of heating at 94°C for 30 seconds followed by 68°C for 44 minutes.

The obtained DNA fragment was cloned using TOPO TA Cloning Kit (Invitrogen) according to the method described in the manual attached thereto. ABI377DNA sequencer was used to read the base sequence of the cloned DNA, to identify the base sequence of 3'-terminus (SEQ ID NO:15).

Primer ZAQL-CF (SEQ ID NO:16) and Primer ZAQL-XR1 (SEQ ID NO:17) were designed according to the information from the base sequence represented by SEQ ID NO:15 and EST(X40467). PCR was conducted using Primer ZAQL-CF and ZQAL-XR1, and Human testis Marathon-Ready cDNA (CLONTECH) as a temple.

ZAQL-CF: 5'-CCACCATGAGAGGTGCCACG-3' (SEQ ID NO:16)
ZAQL-XR1: 5'-CTCGAGCTCAGGAAAAGGATGGTG-3' (SEQ ID NO:17)

The reaction solution was prepared by mixing 1 µl of PfuTurbo DNA polymerase (Stratagene), 5 µl of 10 x PCR buffer attached, 4 µl of 2.5 mM dNTP mixture, 2.5 µl each of 10 µM primer ZAQL-CF and ZAQL-XR1, 5 µl of template DNA and 30 µl distilled water. The reaction was carried out under the conditions: (1) denaturing at 95°C for 1 minute, (2) repeating 40 times a cycle of heating at 95°C for 1 minute followed by 72°C for 1 minute, and (3) heating for a final extention reaction at 72°C for 10minutes. The obtained DNA fragment was cloned using TOPO TA Cloning Kit (Invitrogen) according to the method described in the manual attached thereto. As a result of reading the base sequences of the cloned DNA fragments using ABI377DNA sequencer, it was found that they have 371 bp sequences represented by SEQ ID NO:18 and SEQ ID NO:19, respectively. The plasmid containing the DNA fragment having the base sequence represented by SEQ ID NO:18, was named pHMITA, and the plasmid containing the DNA fragment having the base sequence represented SEQ ID NO:19, was named pHMITG.

E.coli (Escherichia coli) was transformed using Plasmid pHMITA and pHMITG, and the transformants obtained were named E.coli (Escherichia coli) TOP10/pHMITA and E.coli (Escherichia coli) TOP10/pHMITG, respectively. As a result of analyzing the base sequences of the DNA fragments, it was found that the DNA fragment represented by SEQ ID NO:18 has the DNA(SEQ ID NO:28) encoding Human type ZAQ ligand precursor peptide (type A, 105 amino acid residues) represented by SEQ ID NO:22, and that the DNA fragment represented by SEQ ID NO:19 has the DNA(SEQ ID NO:29) encoding Human type ZAQ ligand precursor peptide (type G, 105 amino acid residues) represented by SEQ ID NO:23.

Further, it was found that the base sequence represented by SEQ ID NO:28 and that represented by SEQ ID NO:29 have a typical signal sequence; the DNA having the base sequence represented by SEQ ID NO:28 has the DNA (SEQ ID NO:26) consisting of 258 base pairs and encoding Human type ZAQ

ligand mature peptide (type A, 86 amino acid residue) represented by SEQ ID NO:20; the DNA having the base sequence represented by SEQ ID NO:29 has the DNA (SEQ ID NO:27) consisting of 258 base pairs and encoding Human type ZAQ ligand mature peptide(type G, 86 amino acid residue) represented by SEQ ID NO:21.

Example 5: Production of Human type ZAQ ligand peptide in mammalian cells (1)

(5-1) Construction of the mammalian expression vector for human type ZAQ ligand precursor peptide

The plasmid obtained in Example 4 (pHMITG) was digested with the restriction enzymes EcoRI and XhoI, to obtain the 382 bp DNA fragment (SEQ ID NO:30) comprising cDNA encoding Human type ZAQ ligand precursor peptide.

That is, Plasmid pHMITG was digested with EcoRI and XhoI, and the obtained DNA fragment was electrophoresed with 1.5% agarose gel. The gel segment comprising the 382 bp band stained with cyber green was extracted by a razor. From said gel segment, the DNA fragment was extracted with Gene Clean spin DNA extraction kit (BIO 101). According to the standard method, the obtained DNA fragment was cloned into the mammalian cell expression vector (pCAN618(Figure 11))containing CMV-IE enhancer and chicken beta-actin promoter as an expression promoter at the cleavage site by the restriction enzymes (EcoRI and XhoI). The cloned DNA fragment was sequenced according to the above method, and it was discovered that it has the base sequence represented by SEQ ID NO:30. This mammalian cell expression vector having the DNA encoding Human type ZAQ ligand precursor peptide was named pCANZAQLg2.

(5-2) Introduction of expression vector into COS7 cells

COS7 cells were purchased from ATCC, and the one subcultured by DMEM medium (10% FBS was added) was used. Using the DMEM medium, COS7 cells were inoculated at a population of 1.5 x 10^6 cells/dish on 10 cm Petri's dish, and cultured at 37°C under 5% CO_2 overnight. To 2 μg of the

expression plasmid of Human type ZAQ ligand precursor peptide (dissolved in 2 µl of TE buffer) (pCANZAQLg2), 298 µl of Buffer EC (Effectene transfection reagent, QIAGEN) and 16 µl of enhancer were added. After mixing for one second, the mixture was allowed to stand at a room temperature for 3 minutes. Then, 60 µl of Effectene Trasfection Reagent was further added to the mixture. After mixing for 10 seconds, the mixture was allowed to stand at room temperature for 10 minutes. Then, the supernatant was removed from the cells inoculated the day before, and the cells were washed with 10 ml of DMEM medium once. Then, 9 ml of DMEM medium was added. 1 ml of DMEM medium was added to the plasmid solution, and after mixing, the mixture was added dropwise to the cells. After mixing the whole system, the cells were cultured at 37°C under 5% CO_2 overnight. The cells were washed with 10 ml of DMEM medium twice, and 10 ml of DMEM medium was added. The cells were cultured in an incubator at 37°C under 5% CO_2 overnight. After 2 days, the cultured supernatant was recovered.

(5-3) Partial purification of human type ZAQ ligand precursor peptide from the cultured supernatant of expression COS7 cells

(5-3-1) Preparation of the cultured supernatant of COS7 cells expressing human type ZAQ ligand precursor peptide

The cultured supernatant of COS7 cells expressing human type ZAQ ligand precursor peptide was recovered, and the extraction solution was prepared as follows. First, 1.1 ml of acetic acid was added dropwise to the cell cultured supernatant (about 18.5ml) to adjust the final concentration to 1 M, and the mixture was agitated for an hour. The twice as much volume of acetone was added thereto, and the mixture was agitated for 30 minutes at 4°C. Then, the mixture was centrifuged at 15,000 rpm for 30 minutes (CR26H, 23 rotor: Hitachi System Engineering Co., Ltd) to obtain a supernatant. The obtained supernatant was evaporated to remove acetone, and then lyophilized with a lyophilizer (12EL;VirTis).

(5-3-2) Sephadex G50 Gel chromatography and Sep Pak column chromatography of the cultured supernatant of COS7 cells expressing human type ZAQ ligand precursor peptide

The lyophilized powders obtained in (5-3-1) were dissolved in 2 ml of 1 M acetic acid, and then, loaded onto Sephadex G15 column (3cm x 35ml, Pharmacia Biotech), which was equilibrated with 1 M acetic acid. 1 M of acetic acid was flown through the column. 5 ml each of the eluted solution was taken, and a fraction number was given to each fraction. Then, the each fraction was lyophilized with a lyophilizer (12EL; Virtis).

SepPak C18-5g column (10ml) was swollen with methanol, and equilibrated with 0.1% of trifluoroacetic acid/distilled water. The lyophilized products of Fractions Nos. 1-16 taken from the divided fractions of Sephadex G50 gel chromatography, were dissolved in 3 ml of trifluoroacetic acid/distilled water, and loaded onto SepPak C18-5g column. Then, the column was washed with 24 ml of 0.1% trifluoroacetic acid/distilled water, and eluted with 20 ml of 0.1% trifluoroacetic acid/60% acetonitrile. The eluted solution was subjected to a Savant speed vac concentrator.

(5-3-3) Purification of Super ODS reversed phase high performance liquid chromatography

The column for TSKgel Super ODS reversed phase high performance liquid chromatography (Toso, 0.46cm x 10cm) was equilibrated with Solution A at a flow rate of 1 ml/min at 40°C. After the SepPak C18-5g Column fraction obtained in (5-3-2) was subjected to a Savant speed vac concentrator, loaded onto Super ODS reversed phase high performance liquid chromatography, and eluted with Solution A (0.1% trifluoroacetic acid/distilled water) and Solution B (0.1% trifluoroacetic acid/100% acetonitrile) at a flow rate of 1 ml/min while changing a Solution A/Solution B ratio from 100 vol.% Solution A /0 vol.% Solution B to 0 vol.% Solution A/100 vol.% Solution B for 60 minutes in a linear gradient manner, to recover the eluted solution.

1 ml each of the solution was collected, and a fraction number was given to each fraction. The whole amount of the divided fractions was lyophilized with a lyophilizer. The products were dissolved in 150 µl of a mixture wherein 2.5mM Probenecid and 0.2% BSA were added to H/HBSS. Using this solution, the receptor activation function with respect to ZAQC-B1 cells was measured according to the testing method (5-3-4).

(5-3-4) Measurement of the activity of increasing intracellular Ca ion concentration by FLIPR

The samples obtained in above (5-3-4) were assayed for the activity of increasing intracellular Ca ion concentration in ZAQ expression cells (ZAQC-B1) obtained in Example 3 (3-5) by FLIPR. hOT7T175 expression cells (h0T7T175-16; described in WO00/24890) were used as control.

The ZAQC-B1 cells and h0T7T175 cells that were subcultured in DMEM supplemented with 10% dialyzed fetal bovine serum (hereinafter referred to as d FBS) were used. The ZAQC-B1 cells and h0T7T175 cells were suspended respectively in the medium(10% dFBS-DMEM) to ajust their consentration to 15 x 10^4 cells/ml. 200µl of the cells (3.0 x 10^4 cells/200 µl /well) were inoculated in each well (Black plate clear bottom, Coster) using a dispensing pipet. After culturing at 37°C under 5% CO_2 for one day, these cells were used (hereinafter referred to as cell plate). 21 ml of H/HBSS (HANKS'9.8 g, Sodium bicarbonate 0.35 g, HEPES 4.77 g, adjusted to pH 7.4 with Sodium hydroxide and sterilized with a sterilizing filter), 210 µl of 250 mM Probenecid, and 210 µl of fetal bovine serum (FBS) were mixed. Fluo3-AM2 (50 µg) was dissolved in 42 µl of dimethyl sulfoxide and 42 µl of 20% Pluronic acid. The mixture was added to above h/HBSS-Probenecid-FBS. After mixing, the medium was removed from the cell plate, and 100 µl of the mixture was poured into each well of the cell plate using an 8-well pipet. Then, the cells were incubated at 37°C under 5% CO_2 for one hour (dye loading). With respect to these samples for the assay obtained in above (5-3-3), each

fraction was dissolved in 150 µl of a mixture wherein 2.5mM Probenecid and 0.2% BSA were added to H/HBSS, and transferred to FLIPR 96-well plate (V-Bottom plate, Coster)(hereinafter, referred to as sample plate). After completion of dye loading, the cell plate was washed four times with the washing buffer (H/HBSS with 2.5mM Probenecid) using a plate washer (Molecular Devices) and 100µl of the washing buffer was left for the further usage. This cell plate and the sample plate were loaded onto FLIPR to conduct the assay (0.05 ml of samples were transferred from the sample plate to the cell plate by FLIPR). The activity of increasing intracellular Ca ion concentration, which is specific to ZAQ-B1 cell, was observed in Fractions Nos.48-68. From the above, it was found that the target components having the receptor activity function with respect to ZAQC-B1, i.e., ZAQC activating component, was eluted in Fractions Nos. 48-68.

Example 6: Production of Human ZAQ ligand peptide in mamalian cells (2)

(6-1) Preparation of the cultured supernatant medium

As described in Example 5, the expression plasmid of human type ZAQ ligand precursor peptide (pCANZAQLg2) was introduced into COS7 cells. That is, COS7 cells were inoculated at a population of 3.0×10^6 cells/dish on 15 cm Petri's dish, and cultured at 37°C under 5% CO_2 overnight. 600 µl of Buffer EC (Effectene transfection reagent, QIAGEN) was added to 4 µg of the plasmid (pCANZAQLg2) (dissolved in 4 µl of TE buffer). 32 µl of Enhancer was further added thereto. After mixing for one second, the mixture was allowed to stand for 3 minutes at room temperature. Further, 120 µl of Effectene Transfection Reagent was added. After mixing for 10 seconds, the mixture was allowed to stand for 10 minutes at room temperature. The supernatant was removed from the cells inoculated the day before, and the cells were washed with 10 ml of DMEM medium once. Then, 30 ml of DMEM medium was added. 1 ml of DMEM medium was added to the plasmid solution, and after mixing, the mixture was added

dropwise to the cells. After mixing the whole system, the cells were cultured in an incubator at 37°C under 5% CO_2 overnight. The cells were washed with 10 ml of DMEM medium once. Then, 20 ml of DMEM medium was added. The cells were cultured in an incubator at 37°C under 5% CO_2 overnight. The next day, thecultured supernatant was collected, and 20 ml of DMEM medium was added to the system, and cultured in an incubator at 37°C under 5% CO_2 overnight, to recover the cultured supernatant.

(6-2) Purification of Human type ZAQ ligand peptide from the cultured supernatant

Using the method described in above (6-1), the conditioned medium was recovered from 80 Petri's dishes having a diameter of 15 cm. Acetic acd was added into the medium to adjust the final concentration to become 1 M. After 1 hour of agitation, acetone was added twice volume as much as the solution to precipitate proteins. The solution was agitated for 30 minutes at 4°C. Then, the solution was centrifuged at 10,000rpm for 30 minutes with a high-speed centrifuge (CR26H RR10A type rotor: Hitachi System Engineering Co., Ltd.), to obtain a supernatant. The obtained supernatant was evaporated with an evaporator to remove acetone. The solution was flown into the reversed phase column (Waters C18, 100g) equilibrated in advance with 0.1% trifluoroacetic acid/distilled water (1,000ml). After washing the column with 0.1% trifuluoroacetic acid/distilled water (1,000ml), followed by further washing with 0.1% trifuluoroacetic acid /20% acetonitrile (1,000ml), the peptide was eluted with 0.1% trifuluoroacetic acid/60% acetonitrile (1,000ml). The eluted solution was evaporated, and lyophilized with a lyophilizer (12EL; Vir Tis).

The column for TSKgel ODS80TM reversed phase high performance liquid chromatography (Tosoh, 21.5 mm x 30 cm) was equilibrated with Solution A (0.1% trifluoroacetic acid/distilled water) at a flow rate of 4 ml/min at 40°C. The lyophilized powders obtained were dissolved in Solution A, adsorbed to said ODS80TM column, and then treated with

Solution A (0.1% trifluoroacetic acid/distilled water) and Solution B (0.1 % trifuluoroacetic acid/60% acetonitrile) at a flow rate of 4 ml/min for 120 minutes while changing a Solution A/B ratio from 60 vol.% Solution A /40 vol. % Solution B to 0 vol.% Solution A/100 vol.% Solution B in a liner gradient manner, to elute peptides.

8ml each of the eluted solution was taken separately, and a fraction number was given to each fraction. 50 µl of the solution was taken from the divided fractions, and lyophilized with a lyophilizer (12EL; VirTis). 200 µl of a mixture wherein 2.5 mM Probenecid and 0.2% BSA were added to H/HBSS, were added to the lyophilized product to dissolve it. This solution was used to measure the function of receptor activity against ZAQC-B1 cells according to the above testing method (5-3-4). As a result, it was found that the target component having the function of receptor activity with respect to ZAQC-B1, i.e., ZAQ activating component, was eluted in Fraction No. 32.

The column for TSKgel CM-2SW ion-exchange high performance liquid chromatography (Toso,4.6 mm x 25cm) was equilibrated with Solution A (10mM ammonium formate/10% acetonitrile) at a flow rate of 1 ml/min at 25°C.

Fraction No. 32 was loaded onto CM-2SW column, and eluted with Solution A (10 mM ammonium formate /10% acetonitrile) and Solution B (1000 mM ammonium formate /10% acetonitrile) at a flow rate of 1 ml/min for 60 minutes while changing a Solution A/Solution B ratio from 100 vol.% Solution A /0 vol. % Solution B to 0 vol.% Solution A/100 vol.% Solution B in a liner gradient manner, to elute peptides.

1 ml each of the eluted solution was taken separately, and a fraction number was given to each fraction. 1.5 µl of the solution was taken from the fractions, and diluted with 200 µl of H/HBSS to which 2.5 mM Probenecid and 0.2% BSA were added. This solution was used to measure the function of receptor activation with respect to ZAQC-B1 cells according to the above testing method (5-3-4). As a result, it was found that the target components having the function

of receptor activation with respect to ZAQC-B1, i.e., ZAQC activating component, were eluted in Fractions No. 56 and 57.

The column for TSKgel Super phenyl reversed phase high performance liquid chromatoTabley (Toso,4.6 mm x 30 cm) was equilibrated by Solution A (0.1% trifluoroacetic acid/distilled water) at a flow rate of 4 ml/min at 40°C. Above fractions Nos.56 and 57 were loaded onto said Super phenyl column, and eluted with Solution A (0.1% trifluoroacetic acid/distilled water) and Solution B (0.1 % trifuluoroacetic acid/60% acetonitrile) at a flow rate of 1 ml/min for 60 minutes while changing a Solution A/Solution B ratio from 70 vol.% Solution A/30 vol.% Solution B to 50 vol.% Solution A/50 vol.% Solution B in a liner gradient manner, to elute peptides

1 ml each of the eluted solution was taken separately and a fraction number was given to each faraction. 1.5 µl of the solution was taken from the divided fractions and diluted with 200 µl of a mixture wherein 2.5 mM Probenecid and 0.2% BSA were added to H/HBSS. This solution was used to measure the function of receptor activity with respect to ZAQC-B1 cells according to the above testing method (5-3-4). As a result, it was found that the target components having the function of receptor activation with respect to ZAQC-B1, ZAQC activating components, were eluted in Fractions No. 54, 55 and 56. The activaty was matched with the single ultraviolet absorption peak, and this result was interpreted that the activating component was purified enough to homogeneity.

The solvent of the purified sample of ZAQ activating components was removed by lyophilization. The obtained lyophilized product was dissolved in DMSO (dimethyl sulfoxide). Some portion of this solution (about 7.5 pmol) was used for the analysis of amino acid sequence of N-terminus. As a result, out of the amino acid residues from N-terminal to the 10^{th} amino acid residue, 9 amino acid residues were able to identify (Ala, Val, Ile, Thr, Gly, Ala, Xaa, Glu, Arg, Asp (SEQ ID NO.:31; Xaa was not identified)). The obtained amino acid sequence was matched with the N-

terminus of the amino acid sequence of Human type ZAQ ligand mature peptide. The mass spectrometry was conducted for the purified sample of ZAQ activating component with Finnigan LCQ LC/MC apparatus (Thermoquest, San Jose, Ca) according to the electro spray-ionization method. It was found that the molecular weight was 9657.6. This result was well matched to the theoretical value (9657.3) of the Human type ZAQ ligand mature peptide having the residue in which all of 10 Cystein residues formed disulfide bonds. As a result, it was confirmed that the purified sample of ZAQ activating components has human type ZAQ ligand mature peptide which has the amino acid sequence represented by SEQ ID NO:21.

(6-3) Measurement of the ZAQ activating function of the purified human type ZAQ ligand peptide The function of receptor activation of the human type ZAQ ligand mature peptide purified in above (6-2) with respect to ZAQ B-1 cells was measured according to the above testing method (5-3-4). As a result, human type ZAQ ligand mature peptide caused increase in the intracellular calcium ion concentration in ZAQ expression CHO cells (ZAQC-B1 cells) in a concentration-dependent manner. The value of EC_{50} was 96 pM and it was discovered that human type ZAQ ligand mature peptide shows the strong agaonist activity. The results are shown in Fig. 10.

INDUSTRIAL APPLICABILITY

The protein of this invention, its partial peptides, or salts thereof and the DNA encoding the same can be used for; (i) determination of ligands (agonists); (ii) preparation of antibodies and antisera; (iii) construction of recombinant protein expression systems; (iv) development of the receptor binding assay systems using the expression systems and screening of pharmaceutical candidate compounds; (v) effecting drug design based on comparison with structurally similar ligand receptors; (vi)reagents for preparation of probes and PCR primers for gene diagnosis; (vii) production of transgenic animals; and (viii) pharmaceutical drugs for the gene prophylaxis/therapy.

CLAIMS

1. A protein which comprises the same or substantially the same amino acid sequence as the amino acid sequence represented by SEQ ID NO:1, or a salt thereof.

2. A partial peptide of the protein according to claim 1, or a salt thereof.

3. A DNA which comprises a DNA encoding the protein according to claim 1.

4. A DNA according to claim 3, which is represented by SEQ ID NO:2 or SEQ ID NO:3.

5. A recombinant vector which comprises the DNA according to claim 3.

6. A transformant transformed with the recombinant vector according to claim 5.

7. A method of producing the protein or its salt according to claim 1, which comprises culturing the transformant according to claim 6, and producing and accumulating the protein according to claim 1.

8. An antibody to the protein according to claim 1, the partial peptide according to claim 2, or a salt thereof.

9. A method of determining a ligand to the protein or its salt according to claim 1, which comprises using the protein according to claim 1 or the partial peptide according to claim 2, or a salt thereof.

10. A method of screening a compound or its salt that alters the binding property between a ligand and the protein or its salt according to claim 1, which comprises using the protein according to claim 1, the partial peptide according to claim 2, or a salt thereof.

11. A kit for screening a compound or its salt that alters the binding property between a ligand and the protein or its salt according to claim 1, comprising the protein according to claim 1 or the partial peptide according to claim 2, or a salt thereof.

12. A compound or its salt that alters the binding property between a ligand and the protein or its salt

according to claim 1, which is obtainable using the screening method according to claim 10 or the screening kit according to claim 11.

13. A pharmaceutical composition comprising a compound or its salt that alters the binding property between a ligand and the protein or its salt according to claim 1, which is obtainable using the screening method according to claim 10 or the screening kit according to claim 11.

14. A DNA that hybridizes to the DNA according to claim 3 under highly stringent conditions.

ABSTRACT OF THE DISCLOSURE

The present invention relates to a human-derived protein, its partial peptides, or salts thereof, a DNA encoding the protein, methods for determining a ligand to the protein, screening methods/screening kits for a compound that alters the binding property between a ligand and the protein, a compound obtainable by the screening or its salts, etc.

The human-derived protein of this invention or the DNA encoding the protein can be used for ① determination of ligands to the e present invention; ② prophylactic and/or therapeutic agents for diseases associated with dysfunction of the protein of the present invention; ③ screening of compounds (agonists, antagonists, etc.) that alter the binding property between the protein of the present invention and ligands.

Fig.1

```
        10         20         30         40         50         60
ATGGAGACCACCATGGGGTTCATGGATGACAATGCCACCAACACTTCCACCAGCTTCCTT
M  E  T  T  M  G  F  M  D  D  N  A  T  N  T  S  T  S  F  L

        70         80         90        100        110        120
TCTGTGCTCAACCCTCATGGAGCCCATGCCACTTCCTTCCCATTCAACTTCAGCTACAGC
S  V  L  N  P  H  G  A  H  A  T  S  F  P  F  N  F  S  Y  S

       130        140        150        160        170        180
GACTATGATATGCCTTTGGATGAAGATGAGGATGTGACCAATTCCAGGACGTTCTTTGCT
D  Y  D  M  P  L  D  E  D  E  D  V  T  N  S  R  T  F  F  A

       190        200        210        220        230        240
GCCAAGATTGTCATTGGGATGGCCCTGGTGGGCATCATGCTGGTCTGCGGCATTGGAAAC
A  K  I  V  I  G  M  A  L  V  G  I  M  L  V  C  G  I  G  N

       250        260        270        280        290        300
TTCATCTTTATCGCTGCCCTGGTCCGCTACAAGAAACTGCGCAACCTCACCAACCTGCTC
F  I  F  I  A  A  L  V  R  Y  K  K  L  R  N  L  T  N  L  L

       310        320        330        340        350        360
ATCGCCAACCTGGCCATCTCTGACTTCCTGGTGGCCATTGTCTGCTGCCCCTTTGAGATG
I  A  N  L  A  I  S  D  F  L  V  A  I  V  C  C  P  F  E  M

       370        380        390        400        410        420
GACTACTATGTGGTGCGCCAGCTCTCCTGGGAGCACGGCCACGTCCTGTGCACCTCTGTC
D  Y  Y  V  V  R  Q  L  S  W  E  H  G  H  V  L  C  T  S  V
```

Fig.2

```
       430        440        450        460        470        480
AACTACCTGCGCACTGTCTCTCTCTATGTCTCCACCAATGCCCTGCTGGCCATCGCCATT
N  Y  L  R  T  V  S  L  Y  V  S  T  N  A  L  L  A  I  A  I

       490        500        510        520        530        540
GACAGGTATCTGGCTATTGTCCATCCGCTGAGACCACGGATGAAGTGCCAAACAGCCACT
D  R  Y  L  A  I  V  H  P  L  R  P  R  M  K  C  Q  T  A  T

       550        560        570        580        590        600
GGCCTGATTGCCTTGGTGTGGACGGTGTCCATCCTGATCGCCATCCCTTCCGCCTACTTC
G  L  I  A  L  V  W  T  V  S  I  L  I  A  I  P  S  A  Y  F

       610        620        630        640        650        660
ACCACCGAGACGGTCCTCGTCATTGTCAAGAGCCAGGAAAAGATCTTCTGCGGCCAGATC
T  T  E  T  V  L  V  I  V  K  S  Q  E  K  I  F  C  G  Q  I

       670        680        690        700        710        720
TGGCCTGTGGACCAGCAGCTCTACTACAAGTCCTACTTCCTCTTTATCTTTGGCATAGAA
W  P  V  D  Q  Q  L  Y  Y  K  S  Y  F  L  F  I  F  G  I  E

       730        740        750        760        770        780
TTCGTGGGCCCCGTGGTCACCATGACCCTGTGCTATGCCAGGATCTCCCGGGAGCTCTGG
F  V  G  P  V  V  T  M  T  L  C  Y  A  R  I  S  R  E  L  W

       790        800        810        820        830        840
TTCAAGGCGGTCCCTGGATTCCAGACAGAGCAGATCCGCAAGAGGCTGCGCTGCCGCAGG
F  K  A  V  P  G  F  Q  T  E  Q  I  R  K  R  L  R  C  R  R

       850        860        870        880        890        900
AAGACGGTCCTGGTGCTCATGTGCATCCTCACCGCCTACGTGCTATGCTGGGCGCCCTTC
K  T  V  L  V  L  M  C  I  L  T  A  Y  V  L  C  W  A  P  F
```

Fig.3

```
       910        920        930        940        950        960
TACGGCTTCACCATCGTGCGCGACTTCTTCCCCACCGTGTTCGTGAAGGAGAAGCACTAC
Y  G  F  T  I  V  R  D  F  F  P  T  V  F  V  K  E  K  H  Y

       970        980        990       1000       1010       1020
CTCACTGCCTTCTACATCGTCGAGTGCATCGCCATGAGCAACAGCATGATCAACACTCTG
L  T  A  F  Y  I  V  E  C  I  A  M  S  N  S  M  I  N  T  L

      1030       1040       1050       1060       1070       1080
TGCTTCGTGACCGTCAAGAACGACACCGTCAAGTACTTCAAAAAGATCATGTTGCTCCAC
C  F  V  T  V  K  N  D  T  V  K  Y  F  K  K  I  M  L  L  H

      1090       1100       1110       1120       1130       1140
TGGAAGGCTTCTTACAATGGCGGTAAGTCCAGTGCAGACCTGGACCTCAAGACAATTGGG
W  K  A  S  Y  N  G  G  K  S  S  A  D  L  D  L  K  T  I  G

      1150       1160       1170       1180       1190
ATGCCTGCCACCGAAGAGGTGGACTGCATCAGACTAAAATAA
M  P  A  T  E  E  V  D  C  I  R  L  K  *
```

Fig.4

```
        10         20         30         40         50         60
ATGGAGACCACCATGGGGTTCATGGATGACAATGCCACCAACACTTCCACCAGCTTCCTT
M  E  T  T  M  G  F  M  D  D  N  A  T  N  T  S  T  S  F  L

        70         80         90        100        110        120
TCTGTGCTCAACCCTCATGGAGCCCATGCCACTTCCTTCCCATTCAACTTCAGCTACAGC
S  V  L  N  P  H  G  A  H  A  T  S  F  P  F  N  F  S  Y  S

       130        140        150        160        170        180
GACTATGATATGCCTTTGGATGAAGATGAGGATGTGACCAATTCCAGGACGTTCTTTGCT
D  Y  D  M  P  L  D  E  D  E  D  V  T  N  S  R  T  F  F  A

       190        200        210        220        230        240
GCCAAGATTGTCATTGGGATGGCCCTGGTGGGCATCATGCTGGTCTGCGGCATTGGAAAC
A  K  I  V  I  G  M  A  L  V  G  I  M  L  V  C  G  I  G  N

       250        260        270        280        290        300
TTCATCTTTATCGCTGCCCTGGTCCGCTACAAGAAACTGCGCAACCTCACCAACCTGCTC
F  I  F  I  A  A  L  V  R  Y  K  K  L  R  N  L  T  N  L  L

       310        320        330        340        350        360
ATCGCCAACCTGGCCATCTCTGACTTCCTGGTGGCCATTGTCTGCTGCCCCTTTGAGATG
I  A  N  L  A  I  S  D  F  L  V  A  I  V  C  C  P  F  E  M

       370        380        390        400        410        420
GACTACTATGTGGTGCGCCAGCTCTCCTGGGAGCACGGCCACGTCCTGTGCACCTCTGTC
D  Y  Y  V  V  R  Q  L  S  W  E  H  G  H  V  L  C  T  S  V
```

Fig.5

```
        430        440        450        460        470        480
AACTACCTGCGCACTGTCTCTCTCTATGTCTCCACCAATGCCCTGCTGGCCATCGCCATT
N  Y  L  R  T  V  S  L  Y  V  S  T  N  A  L  L  A  I  A  I

        490        500        510        520        530        540
GACAGGTATCTGGCTATTGTCCATCCGCTGAGACCACGGATGAAGTGCCAAACAGCCACT
D  R  Y  L  A  I  V  H  P  L  R  P  R  M  K  C  Q  T  A  T

        550        560        570        580        590        600
GGCCTGATTGCCTTGGTGTGGACGGTGTCCATCCTGATCGCCATCCCTTCCGCCTACTTC
G  L  I  A  L  V  W  T  V  S  I  L  I  A  I  P  S  A  Y  F

        610        620        630        640        650        660
ACCACCGAGACGGTCCTCGTCATTGTCAAGAGCCAGGAAAAGATCTTCTGCGGCCAGATC
T  T  E  T  V  L  V  I  V  K  S  Q  E  K  I  F  C  G  Q  I

        670        680        690        700        710        720
TGGCCTGTGGACCAGCAGCTCTACTACAAGTCCTACTTCCTCTTTATCTTTGGCATAGAA
W  P  V  D  Q  Q  L  Y  Y  K  S  Y  F  L  F  I  F  G  I  E

        730        740        750        760        770        780
TTCGTGGGCCCCGTGGTCACCATGACCCTGTGCTATGCCAGGATCTCCCGGGAGCTCTGG
F  V  G  P  V  V  T  M  T  L  C  Y  A  R  I  S  R  E  L  W

        790        800        810        820        830        840
TTCAAGGCGGTCCCTGGATTCCAGACAGAGCAGATCCGCAAGAGGCTGCGCTGCCGCAGG
F  K  A  V  P  G  F  Q  T  E  Q  I  R  K  R  L  R  C  R  R

        850        860        870        880        890        900
AAGACGGTCCTGGTGCTCATGTGCATCCTCACCGCCTACGTGCTATGCTGGGCGCCCTTC
K  T  V  L  V  L  M  C  I  L  T  A  Y  V  L  C  W  A  P  F
```

Fig.6

```
      910       920       930       940       950       960
TACGGCTTCACCATCGTGCGCGACTTCTTCCCCACCGTGTTTGTGAAGGAGAAGCACTAC
Y  G  F  T  I  V  R  D  F  F  P  T  V  F  V  K  E  K  H  Y

      970       980       990      1000      1010      1020
CTCACTGCCTTCTACATCGTCGAGTGCATCGCCATGAGCAACAGCATGATCAACACTCTG
L  T  A  F  Y  I  V  E  C  I  A  M  S  N  S  M  I  N  T  L

     1030      1040      1050      1060      1070      1080
TGCTTCGTGACCGTCAAGAACGACACCGTCAAGTACTTCAAAAAGATCATGTTGCTCCAC
C  F  V  T  V  K  N  D  T  V  K  Y  F  K  K  I  M  L  L  H

     1090      1100      1110      1120      1130      1140
TGGAAGGCTTCTTACAATGGCGGTAAGTCCAGTGCAGACCTGGACCTCAAGACAATTGGG
W  K  A  S  Y  N  G  G  K  S  S  A  D  L  D  L  K  T  I  G

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Fig.7

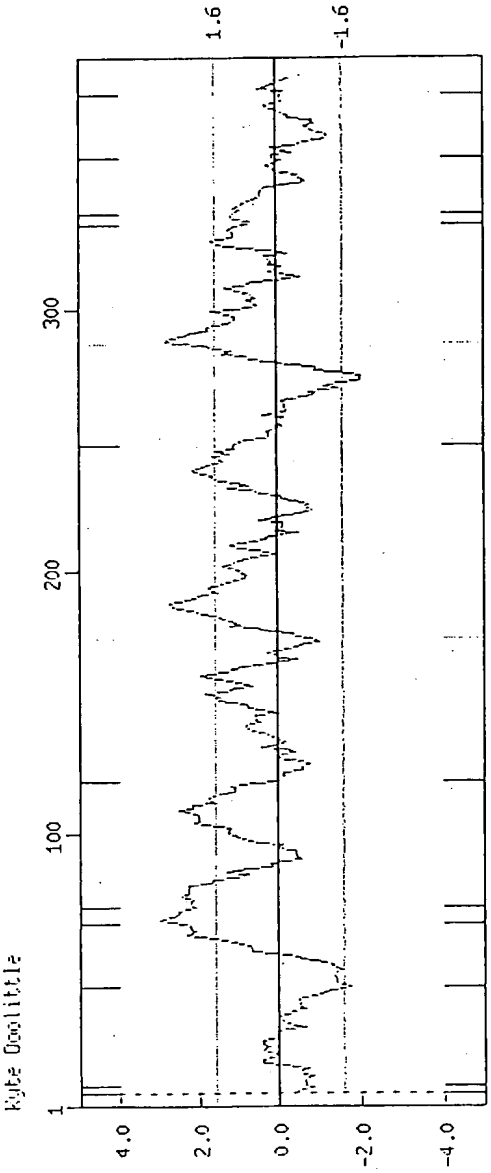
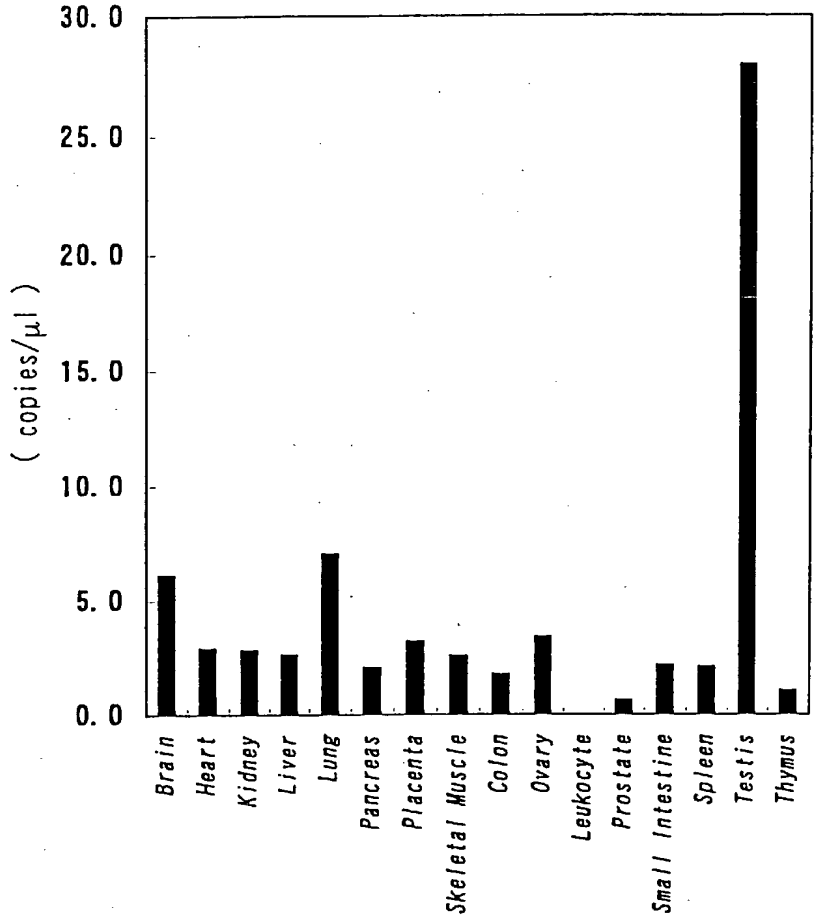


Fig.8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(copies/μl)
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Liver
Lung
Pancreas
Placenta
Skeletal Muscle
Colon
Ovary
Leukocyte
Prostate
Small Intestine
Spleen
Testis
Thymus

Fig.9

```
MIT1          AVITGACERD LQCGKGTCCA VSLWIKSVRV CTPVGTSGED CHPASHKIPF
Human (A type) AVITGACERD VQCGAGTCCA ISLWLRGLRM CTPLGREGEE CHPGSHKIPF
Human (G type) AVITGACERD VQCGAGTCCA ISLWLRGLRM CTPLGREGEE CHPGSHKVPF

MIT1          SGQRMHHTCP CAPNLACVQT SPKKFKCLSK
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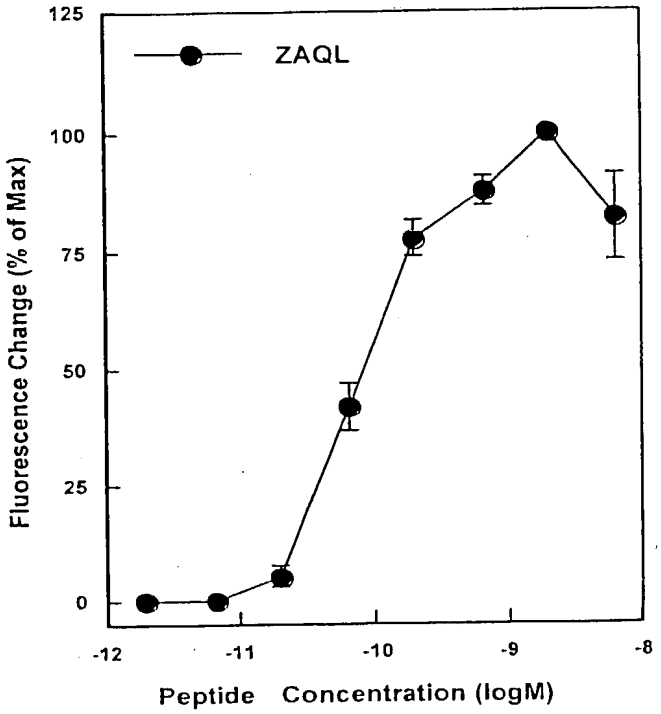
Fig.10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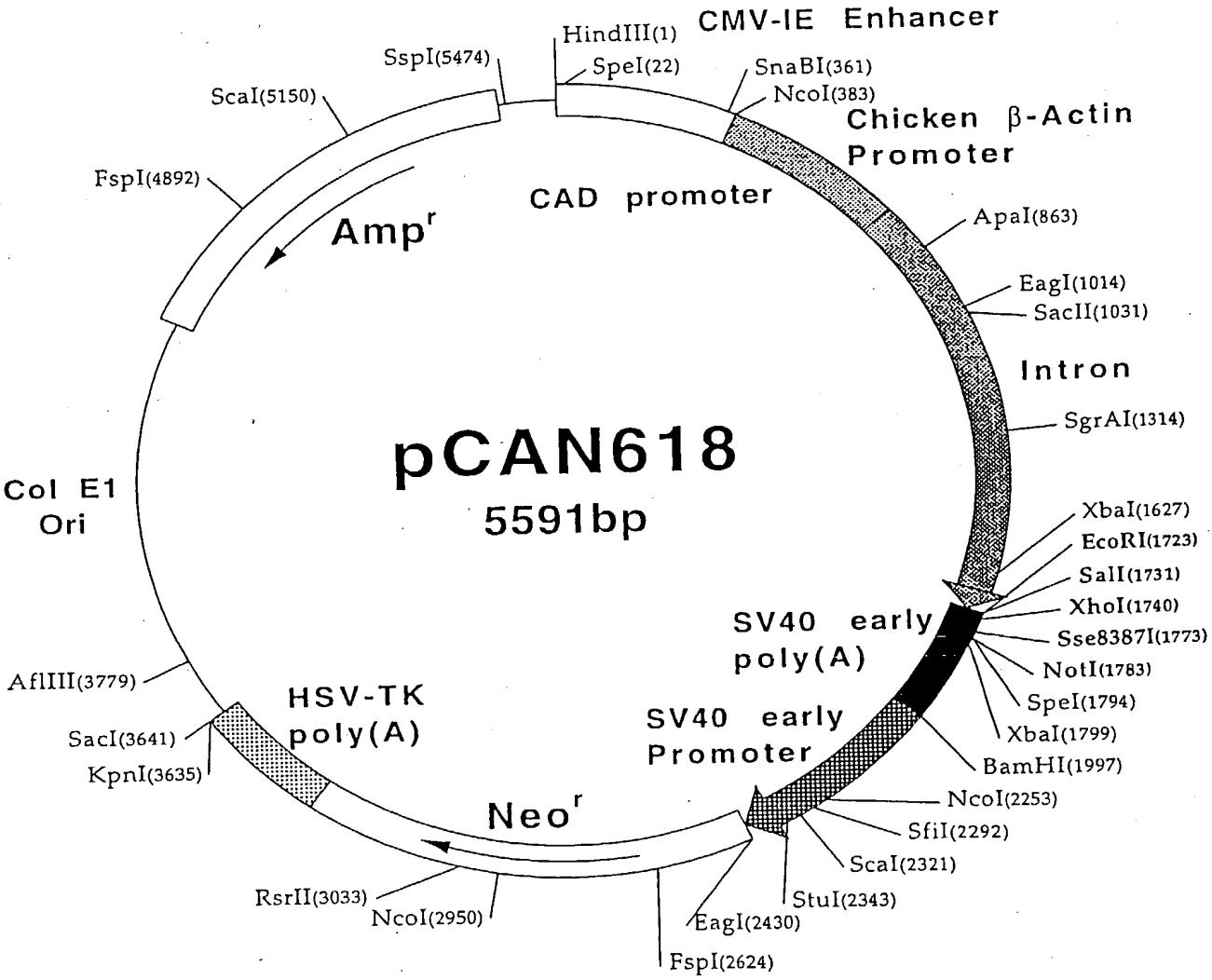
Peptide Concentration (logM)

Fig.11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Neo^r
Col E1 Ori
HindIII(1)
SpeI(22)
SnaBI(361)
NcoI(383)
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EagI(1014)
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XbaI(1627)
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SalI(1731)
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NcoI(2950)
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Express Mail No: EL933048924US
Attorney Docket No. 57127 (46342)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANTS:	T. Watanabe, et al.	EXAMINER:	Not Yet Assigned
U.S.S.N.:	based on PCT/JP00/05684	GROUP:	Not Yet Assigned
FILED:	February 27, 2002		

FOR: NOVEL G PROTEIN-COUPLED RECEPTOR PROTEIN AND DNA THEREOF

BOX NEW PATENT APPLICATION
Commissioner for Patents
Washington, D.C. 20231

Sir:

ASSOCIATE POWER OF ATTORNEY (37 CFR 1.34)

Please recognize the following as an Associate Agent in this case:

Dianne Rees, Reg. No. 45,281.

Respectfully submitted,

Date: February 27, 2002

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130 Water Street
Boston, Massachusetts 02109

Docket No. P01-0258US
57127(46342)

DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that: My residence, post office address and citizenship are as stated below next to my name. I believe I am the original, first and sole inventor (if only one name is listed at 201) below or an original, first and joint inventor (if plural names are listed at 201-208 below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

Novel G Protein-coupled Receptor Protein and DNA Thereof

which is described and claimed in:

☐ the specification attached hereto.

☐ the specification in U.S. Application Serial Number _____, filed on _____.

☒ the specification in PCT international application Number PCT/JP00/05685, filed on August 24, 2000; and was amended on _____.

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a). I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed.

Prior Foreign/PCT Applications and Any Priority Claims Under 35 U.S.C. §119:			
Application No.	**Filing Date**	**Country**	**Priority Claimed Under 35 U.S.C. §119?**
241531/1999	August 27, 1999	JP	x
217474/2000	July 18, 2000	JP	x
			☐YES ☐NO
			☐YES ☐NO

I hereby claim the benefit under 35 U.S.C. §120 of any United States application(s) or PCT international application(s) designating the United States of America that is/are listed below, and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of 35 U.S.C. §112, I acknowledge the duty to disclose material information as defined in 37 CFR §1.56(a) which occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application:

Prior U.S. Applications or PCT International Applications Designating the U.S-Benefit Under 35 U.S.C. §120				
U.S. Applications		Status (Check One)		
Application Serial No.	U.S. Filing Date	Patented	Pending	Abandoned
PCT Applications Designating the U.S.				
Application No.	Filing Date	U.S. Serial No. Assigned		

CLAIM FOR BENEFIT OF PRIOR U.S. PROVISIONAL APPLICATION(S)
(35 U.S.C. §119(e))

I hereby claim the benefit under Title 35, United States Code, §119(e) of any United States provisional application(s) listed below:

Applicant	Provisional Application Number	Filing Date

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) with full powers of association, substitution and revocation to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

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George W. Neuner (Reg. No. 26,964)
Linda M. Buckley (Reg. No. 31,003)
Peter J. Manus (Reg. No. 26,766)
Peter F. Corless (Reg. No. 33,860)
Cara Z. Lowen (Reg. No. 38,227)
William J. Daley, Jr. (Reg. No. 35,487)
Robert L. Buchanan (Reg. No. 40,927)
Christine C. O'Day (Reg. No. 38,256)
Richard E. Gamache (Reg. No. 39,196)
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	RESIDENCE & CITIZENSHIP	CITY	STATE OR FOREIGN COUNTRY	COUNTRY OF CITIZENSHIP
	POST OFFICE ADDRESS	POST OFFICE ADDRESS		

205	FULL NAME OF INVENTOR	LAST NAME	FIRST NAME	MIDDLE NAME
	RESIDENCE & CITIZENSHIP	CITY	STATE OR FOREIGN COUNTRY	COUNTRY OF CITIZENSHIP
	POST OFFICE ADDRESS	POST OFFICE ADDRESS		

[redacted]

I hereby further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Signature of Inventor 201 Takuya Watanabe	Date: Dec. 13, 2001
Signature of Inventor 202 Yasuko Terao Yasuko TERAO	Date: December 13, 2001
Signature of Inventor 203 Yasushi Shintani	Date: Dec. 14, 2001
Signature of Inventor 204	Date:
Signature of Inventor 205	Date:

SEQUENCE LISTINGS

<110> Takeda Chemical Industries, Ltd.

<120> Novel G Protein Coupled Receptor Protein and Its Use

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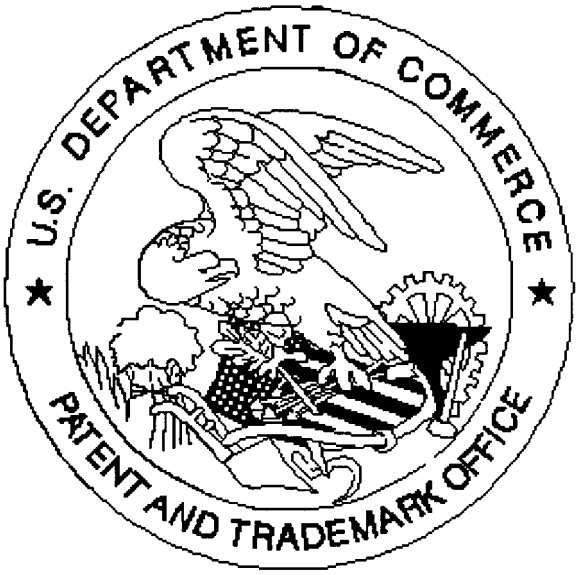

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